# University of Cincinnati

**Date: 6/26/2015**

**I, Divya Ramchandani , hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Pharmaceutical Sciences/Biopharmaceutics.**

It is entitled:

**A Study of Genetic Alterations in Cancer Progression**

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16270

# *A Study of Genetic Alterations in Cancer Progression*

A dissertation submitted to the

Graduate School

of the University of Cincinnati

in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

in the Division of Pharmaceutical Sciences

of the James L. Winkle College of Pharmacy

June 2015

by

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## Abstract

Cancer is one of the most complex diseases, the cause of which is only partially understood and that makes it difficult to identify proper strategies for its treatment. In simple terms, it is defined as an uncontrolled growth of abnormal cells in the body that may spread from their primary site of origin to distant, secondary organs in the body. This spreading of cancer, called cancer metastasis, makes its treatment even more difficult and is responsible for the majority of deaths due to the disease. A multitude of genetic alterations are responsible for tumor invasion and metastasis that form the life-threatening aspect of cancer. There is an incomplete understanding of specific genetic targets for tumor metastasis. Genetic alterations leading to activation of oncogenes and consecutively metastasis genes leads to tumorigenesis and tumor progression, respectively.

While oncogenes have the potential to initiate tumor cell growth and promote their survival, it is the metastasis genes that govern tumor progression and its aggressiveness. Metastasis Genes undergo genetic alteration by two mechanisms: 1. Aberrant Expression; and 2. Alternative Splicing. Both osteopontin (OPN) and tissue factor (TF) are metastasis genes that are known to influence tumor aggressiveness and cause a worsened prognosis in cancer patients.

Alternative splice variants of OPN and their roles in cancer progression have been well characterized (Chae et al., 2009; He et al., 2006). However, the role of OPN genetic polymorphisms in tumor aggressiveness remains unknown. Polymorphism, specifically in the promoter region of a gene, affects the expression levels of its protein product, and hence, may influence the various functions. Via single nucleotide polymorphism (SNP) analysis of DNA

samples from breast cancer patients, we have identified an important polymorphic site in the promoter region of OPN gene that has significant association with breast cancer aggressiveness.

The second mechanism by which genetic alterations affect metastasis genes is via alternative splicing (Brinkman, 2004). After transcription, the primary transcript may undergo splicing to form diverse mRNAs, which may have different properties than the original transcription product of the gene. The primary transcript of Tissue Factor, the main trigger of coagulation, undergoes alternative splicing that yields a secreted variant, termed asTF (alternatively spliced Tissue Factor). asTF does not have a physiologic function in hemostasis, but its expression positively correlates with advanced tumor stages. Since the expression of asTF is prominent in the advanced stages of cancer that are more hypoxic, acidic and nutrient-deficient, we aimed to analyze the mechanism by which asTF might be contributing to tumor progression in such hypoxic conditions. Carbonic anhydrase IX (CAIX) is a novel downstream mediator of the asTF effects in pancreatic cancer progression under hypoxic conditions that model advanced stage tumor micro-environment.

This research has laid the groundwork for the study of the two types of genetic alterations observed in metastasis gene, in the context of OPN and asTF. We hope it will play a significant role in the development of more robust strategies for targeting these genetic anomalies in cancer.

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Chapter 3 contains an original research article:

Ramchandani D, Weber GF. An Osteopontin Promoter Polymorphism is Associated with Aggressiveness in Breast Cancer. Oncology Reports. 2013 Oct; 30(4):1860-8.

**Acknowledgements**

*"It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness, it was the epoch of belief, it was the epoch of incredulity, it was the season of Light, it was the season of Darkness, it was the spring of hope, it was the winter of despair, we had everything before us, we had nothing before us"* – Charles Dickens, *A Tale of Two Cities*

These opening lines from *A Tale of Two Cities* very closely describe my journey towards my Doctor of Philosophy Degree. It would have been a fairly unachievable task to be at this moment, writing "acknowledgements" section of my Dissertation, after years of hard work, without the help of the many wonderful people in my life who deserve the mention here.

First and foremost, I want to acknowledge my advisor, Dr. Georg F. Weber, who has been a great mentor for all these years. He has been instrumental in my development as a Research Scientist and has been invaluable in my training. I owe special thanks for his patience with his guidance on experiment design, data analysis, manuscript preparation and being available always for answering my questions. I also want to thank my committee members for their support and thoughtful critiques of my research. Dr. Bogdanov's guidance and insights have been indispensable. My small sessions with Dr. Desai have always proved to be really fruitful. Dr. Khan and Dr. Hao have really helped me build a better Dissertation with all their ideas and thoughtful inputs. I'm also grateful to Dusten in Dr. Bogdanov's group for all his patience and assistance with animal work.

These five years would have probably proved to be too long, if it wasn't for Abha, my sister. I had had the most fun time when we were together in this city. I do not have enough words to thank my parents and my grandfather for all their blessings and for being the best mentors and

guides as I was growing up. I'm also grateful to Rohit, for being an indispensable part of our lives.

Harsh, thanks for being the amazing person that you are. Even after being mostly at the receiving end of my temper, you would remain sweet and calm. You have been brave enough to stand by me at my most crucial time and have truly been my "rock" for mental & emotional support.

Sudhir, thanks for hanging in with me for the major part of grad school and always speaking the required encouraging words so very timely. I cannot thank you enough just for being there.

I'm grateful to Cincinnati, because it gave me such wonderful friends. Even after being far away from home, you guys would never let me feel alone. Gopi (for being just Gopi!), Preeti (your anecdotes have always been entertaining), Kirthi (for being the wonderful host that you are), Kriti (for being my gym partner even when I would be irregular), Qian (for being so sweet and understanding). Nina, Jai, Swati, Hari, Kiran, Dhananjay: you have all been great friends and thank you Aashutosh, for being there to hear me out for over two decades now. Additionally, I am also grateful to Dr. Harshita Kumari for being such a great friend and guide at so many levels.

And finally, the concluding remarks would be incomplete without the mention of my ever favorite author. Today, I want to thank you for these amazing words that you once said *"It is impossible to live without failing at something, unless you live so cautiously that you might as well not have lived at all - in which case, you fail by default."* ~ J.K. Rowling. With all the ups-and-downs of life, the words and actions of this all-time inspiring author have always kept me going. As I complete this near half a decade journey, I would just like to say *Mischief Managed!*

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## C. List of Abbreviations

| | |
|---|---|
| OPN | Osteopontin |
| TF | Tissue Factor |
| asTF | Alternatively Spliced variant of Tissue Factor |
| CAIX | Carbonic Anhydrase IX |
| NCI | National Cancer Institute |
| CDK | Cyclin Dependent Kinase |
| PDGF-B | Platelet-Derived Growth Factor-B |
| FGF | Fibroblast Growth Factor |
| TGF-α | Transforming Growth Factor-α |
| SHH | Sonic Hedgehog |
| E2F | Eukaryotic Transcription Factor |
| PTEN | Phosphatase and Tensin homolog |
| NF | Neurofibromatosis |
| Rb | Retinoblastoma |
| VHL | von Hippel-Lindau |
| TSC | Tuberous Sclerosis Complex |
| NCAM | Neural Cell Adhesion Molecule |
| PECAM | Platelet Endothelial Cell Adhesion Molecule |
| ICAM | Inter-cellular Adhesion Molecule |
| VCAM | Vascular Cell Adhesion Molecule |
| DNA | Deoxyribonucleic Acid |
| HIF | Hypoxia-Inducible Factor |
| bHLH | Basic Helix-Loop-Helix |
| ODDD | Oxygen-Dependent Degradation Domain |
| PHD | Prolyl Hydroxylases |
| FIH | Factor Inhibiting HIF-1 |
| CBP | CREB-binding Protein |
| VEGF | Vascular Endothelial Growth Factor |
| EPO | Erythropoeitin |
| TSP | Thrombospondin |
| EGF | Epidermal Growth Factor |
| MMP | Matrix Metalloproteinases |
| flTF | Full-Length Tissue Factor |
| FVII | Factor VII |
| FVIIa | Activated Factor VII |
| FX/FXa | Factor X/ activated Factor X |
| LPS | Lipopolysaccharide |
| TNF-α | Tumor Necrosis Factor-α |

| | |
|---|---|
| egr-1 | Early Growth Response Protein 1 |
| TFPI | Tissue Factor Pathway Inhibitor |
| IL | Interleukin |
| CXCL | chemokine (C-X-C motif) ligand |
| PAR | Proteinase-activated Receptor 1 |
| ESE | Exonic Splicing Enhancer |
| FAK | Focal Adhesion Kinase |
| PI3K | Phosphoinositide 3 Kinase |
| MAPK | Mitogen-activated Protein Kinase |
| SPP1 | Secreted Phosphoprotein 1 |
| PCR | Polymerase Chain Reaction |
| SNP | Single Nucleotide Polymorphism |
| RNA | Ribonucleic Acid |
| CATT | Cochran-Armitage Trend Test |
| ER | Estrogen Receptor |
| PR | Progesterone Receptor |
| HER2 | Human Epidermal growth factor Receptor 2 |
| RT-PCR | Real Time- Polymerase Chain Reaction |
| CML | Chronic Myeloid Leukemia |
| ABI | Applied Biosystems |
| MAF | Minor Allele Frequency |
| NCBI | National Center for Biotechnology Information |
| SDS PAGE | Sodium dodecyl sulfate -Polyacrylamide gel electrophoresis |
| PVDF | Polyvinylidene fluoride |
| HRP | Horseradish Peroxidase |
| FBS | Fetal Bovine Serum |
| ECL | Electrochemiluminescence |
| polyHEMA | Poly(2-hydroxyethyl methacrylate) |
| DMSO | Dimethyl sulfoxide |
| U-104 | 4-[[[(4-Fluorophenyl)amino]carbonyl]amino]-benzenesulfonamide |
| PDAC | Pancreatic Ductal Adenocarcinoma |

# Chapter 1
# Introduction

# 1. Introduction

As defined by National Cancer Institute (NCI) "Cancer is the name given to a collection of related diseases. In all types of cancer, some of the body's cells begin to divide without stopping and spread into surrounding tissues." (http://www.cancer.gov/cancertopics/what-is-cancer) Cancer is a name given to a group of diseases that all have one common feature, that is, growth of abnormal cells in the body. These cells are transformed from their normal counterparts in the fact that they are not able to perform the necessary functions for homeostasis as their normal predecessors. Tumors may be benign, which do not spread, or malignant, which can spread into, or invade, nearby tissues. In addition, as these tumors grow, some cancer cells can break off and travel to distant places in the body through the blood or the lymph system and form new tumors far from the original tumor. Many cancers form solid tumors, which are masses of tissue. Cancers of the blood, such as leukemias, generally do not form solid tumors.

## *1.1. Tumorigenesis and Tumor Progression*

The process of tumorigenesis and tumor progression is not well-defined. But for majority of cancers, it can be subdivided into various steps, as depicted in figure 1.1.

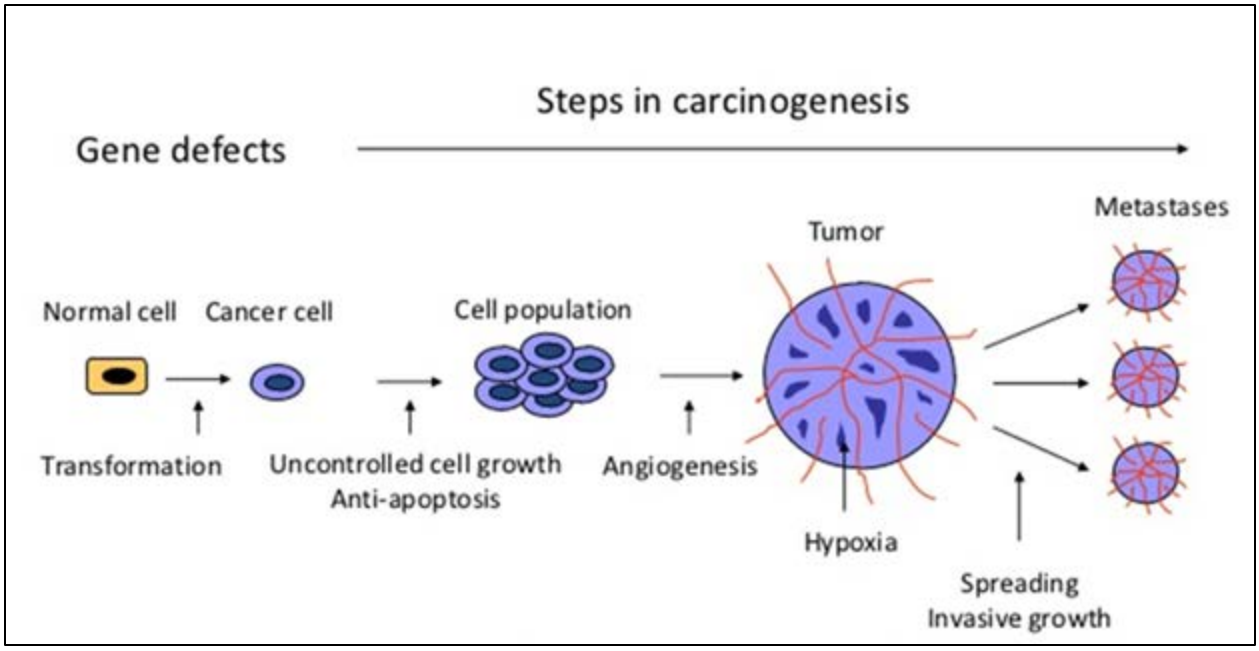


**Figure 1.1 Tumorigenesis and Tumor Progression.** A genetic mutation leads to the transformation of a normal cell to a cancer cell. The transformed cell has the ability to undergo uncontrolled growth and evade cell death. This uncontrolled growth is followed by changes in tumor microenvironment that leads to selection and ultimately dissemination of more aggressive cancer cells, leading to metastasis {Adapted from (Lyng, 2010)}.

A healthy cell, upon a genetic mutation, undergoes transformation to form the cancer cell. These transformed cells are able to evade the normal cell cycle checkpoints to undergo a continuous, uncontrolled cell division so as to multiply. As tumor cells multiply, they outgrow the diffusion limits of oxygen, thus, becoming hypoxic. Because of increased glycolysis, more lactic acid is generated which makes the lesions acidic. Even though new blood vessels are formed in cancer angiogenesis, they are disorganized and cannot effectively alleviate hypoxia (Carmeliet and Jain, 2000). The cells then undergo apoptosis (cell death) or adapt to a glycolytic phenotype. This decrease in pH leads to a selection of motile cells that breach the basement membrane and cause metastasis, perpetuating a vicious circle (Gatenby et al., 2007).

Except for anchorage-independent blood cells and activated stem cells, when cells detach from the extracellular matrix, they undergo programmed cell death, termed anoikis. Cancer cells

acquire the property of anchorage-independence after being released form the primary tumor to survive in the circulation and in secondary sites. This process is inefficient. In the case of transformed cells, less than 1 in every 10,000 cells that detach from a primary tumor, can survive in the circulation before it can form distant metastases. These anchorage-independent cells are able to invade through the primary organ of origin, survive through the lymphatic or blood circulation and invade distant, secondary sites to form metastases.

### *1.2.Genetic Alterations in Cancer*

The process of transformation of normal cells to cancer cells involves one or several mutations in the genetic makeup of the organism. Theses mutations lead to dysregulation of the functions of various gene products and cellular processes. The most prevalent characteristic of cancer cells involves their uncontrolled growth and proliferation. This occurs due to mutations in the genes regulating cell division and cell cycle checkpoints, and may also occur due to aberrations in cell aging and cell death. While the uncontrolled growth of abnormal cells in the body leads to the formation of cancer, a worse prognosis in cancer patients arises due to metastases. Genes that regulate the breach of the contact of cancer cells from their basement membrane, their entry into the circulation system and finally their invasion into secondary sites are known as metastasis genes. Apart from genes that regulate cell growth and spread of the transformed cells, several genes impact the microenvironment of the tumor mass. For instance, vimentin, lactate dehydrogenase B, CD59, CDK2AP1 etc., are only associated with tumor angiogenesis and not with angiogenesis in normal tissues (van Beijnum et al., 2006).

#### 1.2.1. Oncogenes

Transformations that alter gene products and their functions in the first step of tumorigenesis include: i. Gain-of-function mutations of proto-oncogenes that are associated with growth factors

and lead to activation of processes linked to excessive cell divisions. ii. Loss of function mutations in tumor suppressor genes lead to the elimination of cell cycle check points or evasion of cell death. Oncogenes can be categorized into (i) growth factors (PDGF-B, FGF, TGF-α, shh, wnt-1,2, etc.), (ii) growth factor receptors (erbA, erbB, fms, androgen receptor, etc.), (iii) protein kinases (ros, met, trk, erbB2, c-kit, abl, src, raf-1, pkc, PI3K, etc.), (iv) GTP-binding proteins (N-Ras, K-ras, bcl-2, etc.), (v) cyclins-CDKs (cyclin A, cyclin D1, bcl-1, cdk4, cdc25A, cdc25B, mdm2 etc.), (vi) transcription factors (jun, fos, c-myc, E2F, rel, ec.) and (vii) other molecules (β-catenin, hox11, bmi-1, etc.) (Weber, 2007). The different types of tumor suppressor genes can be grouped as follows: (i) receptors (rarα, ptc, dcc), (ii) molecules associated with inactivation of G-Protein-GTP signal (nf-1, tsc2, rassf1A, rcc1), (iii) molecules associated cytoskeleton structure (apc, nf2, PTEN, etc.), (iv) molecules involved in Cyclin-CD activity blockade (p21, p27, p16, cdkn2B, etc.), (v) transcription factors (p53, rb1, vhl, wt1, p73, smad4, ml, etc.), (vi) inducers of cell death (bax, beclin, dapk, fox), ad (vii) miscellaneous (riz1, tsc1, men1, ext1, syk, s100A2 hic-1) (Weber, 2007).

### 1.2.2. Metastasis Genes

Metastasis Genes are stress response genes. These are a unique group of biomolecules, known to be developmentally non-essential, that are associated with the malignancy seen in cancer. The process of migration and invasion is initiated by the binding of homing receptors to their cognate ligands. The locomotion is manifested by the conversion of the energy stored in ATPs into motion which takes place via alternate adhesion and deadhesion of molecules associated with adhesion contacts. The various molecules associated with aggressiveness include: i. extracellular matrix degrading enzymes (MMPs, Plasminogen activation system, Cathepsins, Kallikreins, etc.); ii. Homing receptors and their ligands (selectins, NCAM, PECAM, ICAM, VCAM,

integrins, CD44, chemokine receptors, etc.) and associated signaling molecules. Metastasis suppressor genes counteract the effects of metastasis genes. These include: i. tissue inhibitors of metalloproteinases; ii. Serpins; iii. Cystatins; iv. Cadherins; v. modulators of integrin functions (TM4SF, SHPS); vi. Sermaphorins; vii. others, such as KiSS-1, DMBT1, BRMS-1, NM-23, AKAP-12, MAP2K4, etc.

### 1.2.3. Senescence suppresser genes

Senescence is the phenomenon that limits the number of cell divisions that a somatic cell may undergo. The only exceptions to the process of senescence are the stem cells and germline cells. In order to achieve immortalization, cells must overcome multiple proliferative checkpoints and develop a telomere maintenance mechanism to counteract cell degradation due to the end-replication problem. Transformed cells maintain their telomeres by either a telomerase-dependent or a telomerase-independent mechanism, called Alternative Lengthening of Telomeres (ALT). According to the literature, ALT involves homologous recombination-mediated DNA replication and MRE11/RAD50/NBS1 recombination complex (Cesare and Reddel, 2010). Other mechanisms that affect senescence in genes include: i. Accumulation of reactive oxygen species (Harman, 1956); ii. Cross-linking of proteins, DNA and other structural entities (Abrams et al., 1995); iii. Somatic mutations in mitochondrial DNA (Wei, 1998); iv. levels of plasma cortisol (Van Cauter et al., 1996). In transformed cells, senescence suppressors, like antioxidant thioredoxin etc. (Nakamura et al., 2000), may be upregulated and thus the cells are able to undergo unrestricted cell divisions.

## *1.3. Tumor Hypoxia*

Tumor progression occurs by both genetic alterations within the transformed cells and variable influence of the tumor microenvironment. The genetic aspect of cancer progression is not only

governed by the activity of oncogenes and metastasis genes, but also by the microenvironmental aspect of the stroma surrounding the tumors.

As tumor cells multiply, they outgrow the diffusion limits of oxygen, thus, becoming hypoxic. Because of increased glycolysis, more lactic acid is generated which makes the lesions acidic. Even though new blood vessels are formed in cancer angiogenesis, they are disorganized and cannot effectively alleviate hypoxia (Carmeliet and Jain, 2000). The cells then undergo apoptosis (cell death) or adapt to a glycolytic phenotype. This decrease in pH leads to a selection of motile cells that breach the contact with basement membrane and cause metastasis, perpetuating a vicious circle (Gatenby et al., 2007).

Cell culture conditions for cancer cells have historically been devised to maximize cell growth. Yet, a predisposition to suboptimal, hypoxic and hypoglycemic conditions in advanced tumors has been known for over two decades (Vaupel and Mayer, 2007), but has rarely been included in experimental setups. Because the conventional cell culture systems may not properly assess the pathophysiologic metabolic surroundings, we designed conditions that mimic either the normoxic environment of early stage tumors or the hypoxic environment of late stage tumors to assess the roles of oncogenes or metastasis genes in facilitating tumor progression in the advanced stage microenvironment.

### 1.3.1. Hypoxia Signaling

Due to the uncontrolled and excessive proliferation of cancer cells, there is an imbalance between oxygen demand and supply to the tumor cells that leads to limited oxygen availability to the tumor cells and the surrounding environment. This oxygen limitation plays a key role in controlling neovascularization, glucose metabolism, cell survival and invasion. Hypoxia-

inducible factor (HIF) is the master regulator of these pleiotropic effects under the conditions of oxygen and nutrient stress (Pouysségur et al., 2006).

HIF is activated as oxygen concentration approaches 5% (40 mm Hg) and it progressively increases its expression and activity as the conditions become more hypoxic or anoxic. HIF belongs to the family of basic-helix–loop–helix (bHLH) proteins and is a heterodimer, consisting of an oxygen-dependent α-subunit (HIF-1α, HIF-2α or HIF-3α) and a constitutively expressed β-subunit. Activation of the pleiotropic HIF effects involves stabilization of the α-subunit, followed by its nuclear translocation, hetero-dimerization of α- and β- subunits, binding to the DNA and activation of target genes (Brahimi-Horn et al., 2005; Semenza, 2004). The target genes to which HIF binds consists of hypoxia-response elements (HREs), with the minimal core sequence 5′-RCGTG-3′, which are adjacent to auxiliary motifs specifying the responsive genes.

HIF-α stabilization depends on oxygen-sensing via 2-oxoglutarate- and iron- dependent dioxygenases and via asparaginyl hydroxylase or also referred to as factor inhibiting HIF-1 (FIH). 2-oxoglutarate- and iron- dependent dioxygenases consists of non-heme oxidizing enzymes that directly sense $pO_2$ cellular levels (Berra et al., 2006). PHDs or prolyl hydroxylases hydroxylate two prolyl residues (402 and 564) on HIF-1α that form the oxygen-dependent degradation domain (ODDD). This hydroxylation leads to polyubiquitination via von Hippel–Lindau (VHL) and finally proteasomal degradation of HIF-α subunit (Kaelin, 2003; Maxwell et al., 2001); (Berra et al., 2001; Kallio et al., 1999) (Figure 1.2).

Asparaginyl hydroxylase (or FIH) hydroxylates the asparagine residue at N803 in the C-terminal transcriptional activation domain of HIF-1α. This modification inhibits its interaction with

transcriptional coactivators (p300,CBP), thus, nullifying HIF-α activation and effects in the presence of oxygen (Lando et al., 2002).

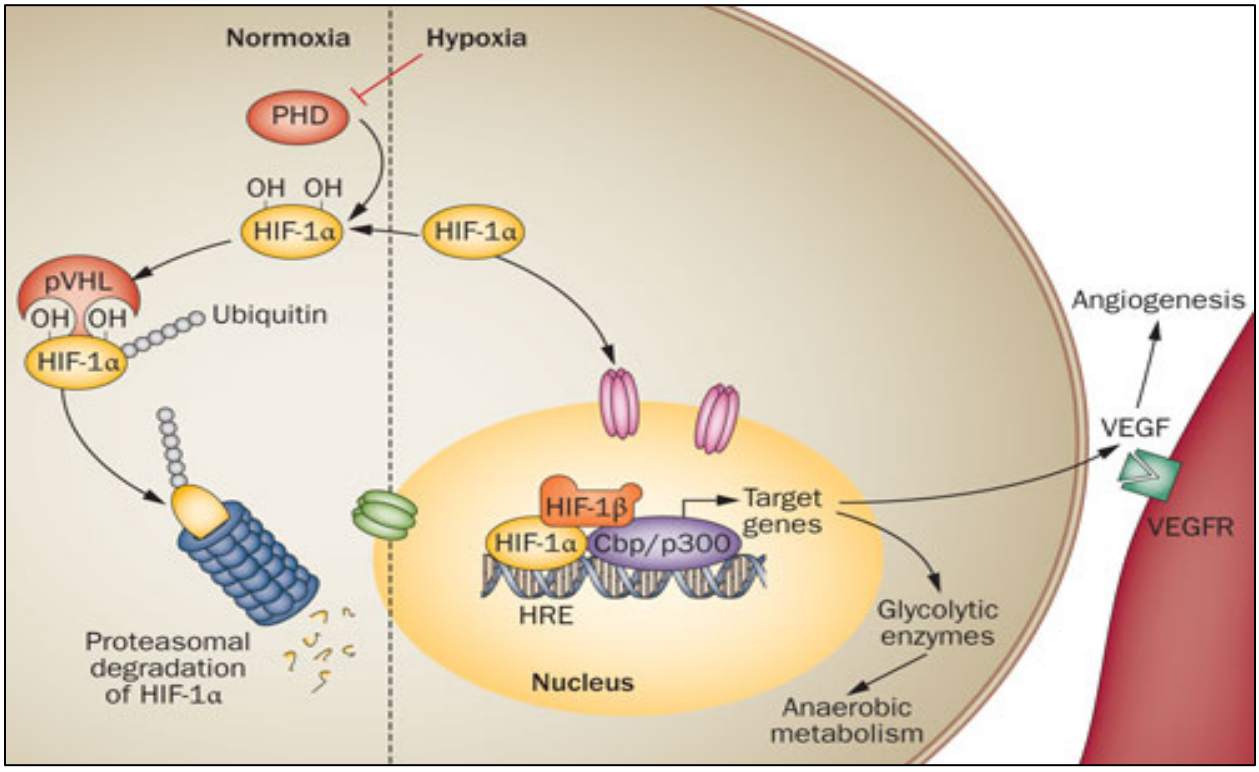


**Figure 1.2 Stabilization of HIF-1α.** HIF-α subunit is degraded under normoxia via PHD-dependent proteasomal degradation. Under hypoxia HIF-α expression is stabilized, undergoes nuclear translocation, dimerize with HIF-β and ultimately induces expression of several hypoxia-regulated genes. (Maes et al., 2012)

### 1.3.2. Hypoxia Genes

Due to hypoxic stress, cells encounter various genomic, proteomic as well as metabolomic changes. These changes affect various functions of cells including their survival, growth, metabolism and apoptosis. Figure 1.3 summarizes the various cellular functions and the corresponding genetic expressions that may be affected under hypoxic stress.

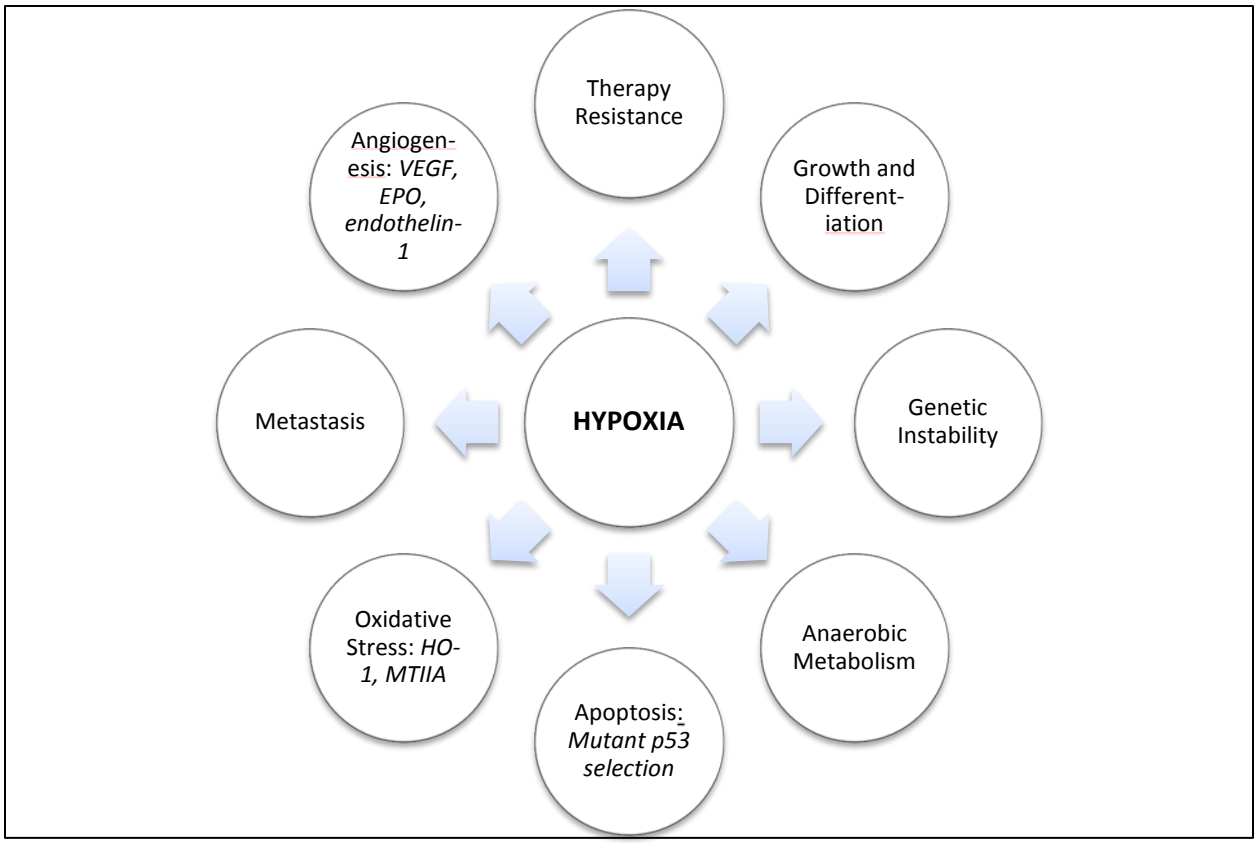


**Figure 1.3 Hypoxia-regulated gene expressions.** In order to adapt to hypoxic stress and develop an aggressive phenotype, expression of several genes is regulated by HIFs. These include genes that regulate glycolytic metabolism, promote proliferation, induce or evade apoptosis, obtain unlimited replication potential & genomic instability, induce angiogenesis, and invade and metastasize {Adapted from (Sutherland, 1998)}.

As described in the figure, a multitude of genes and functions are affected by hypoxia. In the presence of hypoxia, both the proto-oncogene c-jun and the tumor suppressor gene p53 are over-expressed. While c-jun mediates functions like cell growth, differentiation and stress-response, p53 regulates mitogenesis, growth arrest and cell apoptosis. Under hypoxic stress, there is a slow accumulation of mutant p53 in cancer cells over wild-type. This leads to a subsequent dominance of the mutant tumor cell population. Paradoxically, hypoxic stress also leads to an increase in the expression of mediators of neoangiogenesis, especially VEGF. Erythropoeitin (EPO) is also

known to be induced by HIF. Transient expression of thrombospondin-1 (TSP-1), an anti-angiogenic protein, is also increased under hypoxia in the cells expressing the wild-type p53.

Hypoxic stress increases anaerobic metabolism in cells by increasing the levels of associated metabolic enzymes, to maintain energy. Hypoxia also regulates the expression of enzymes or molecules that are effective against cell survival under oxidative stress due to reactive oxygen species. Various growth factors, their receptors and transcription factors are also upregulated under hypoxia including PDGF β, TGFβ, endothelin-1, interleukin 1-α, Flt-1 receptor and EGF receptor. Proteins important for cell survival after radiation or chemotherapy are also known to be over-expressed under hypoxia and these include: glucose-related proteins, heme oxygenase, metallothionein, etc.

### *1.4.Osteopontin*

Metastasis is a complex process involving the interplay of several proteins and enzymes. The genes that encode for such molecules are together celled metastasis genes and include homing receptors, their ligands, associated signaling molecules and extracellular matrix degrading enzymes. These genes are responsible for the invasiveness, anchorage-independent growth & survival of transformed cells, and also determine the site of metastases formation. These genes are developmentally non-essential but are physiologically responsible for processes like wound healing, inflammation and stress-dependent angiogenesis (Ashkar et al., 2000; Weber and Ashkar, 2000a, b).

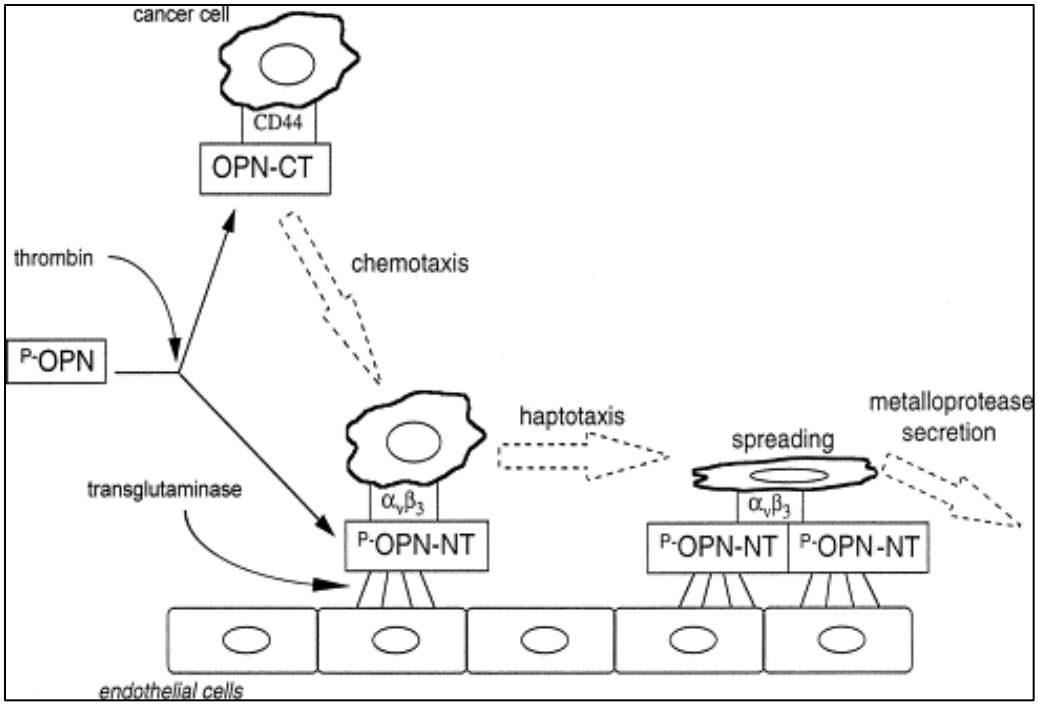


**Figure 1.4 Regulation of Invasion by Osteopontin.** Thrombin cleavage of OPN releases its two receptor binding domains. The C-terminal piece mediates chemotaxis of cells via CD44 receptor at the cleavage site, independent of phosphorylation. The N-terminal piece binds to the integrin receptor to mediate phosphorylation-dependent cell crawling or haptotaxis (Weber, 2001).

Osteopontin (OPN) is one such cytokine that acts as a metastasis gene in multiple malignancies including breast cancer, brain cancer, pancreatic cancer, etc. these genes are known to be deregulated in cancer via aberrant expression and splicing (Weber, 2008). Monocytes, T-lymphocytes, macrophages and vascular smooth muscle cells are all known to secrete OPN. OPN is known to bind to CD44 through its C-terminal domain. The OPN-CD44 interaction induces chemotaxis. The chemoattractant activity of OPN is thus mediated via its C-terminal. On the other hand OPN-integrin αvβ3 interaction takes place through the N-terminal of OPN and is responsible for cell crawling or haptotaxis (Figure 1.4).

### 1.4.1. Osteopontin Protein

OPN is encoded by the SPP1 gene located on chromosome 4q22.1. It consists of six translated exons (figure 1.5). It is an acidic glycoprotein due to the presence of poly-aspartate sequence at the N-terminal domain of the molecule. The middle part of OPN is sensitive to cleavage by various proteinases, most notably thrombin. The cleavage at this site splits the protein into two: i. the C-terminal fragment binds to the homing receptor CD44 (smallest functional domain spans aa 169 to 220), independent of any post-translational modifications and mediates chemotaxis. This OPN fragment also consists of two heparin binding sites (170 and 300) which may form a bridge to the heparin binding site on CD44v3. ii. The N-terminal fragment contains the integrin binding GRGDS motif that binds to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (smallest functional domain spans from aa 71 to 169), in a phosphorylation-dependent manner.

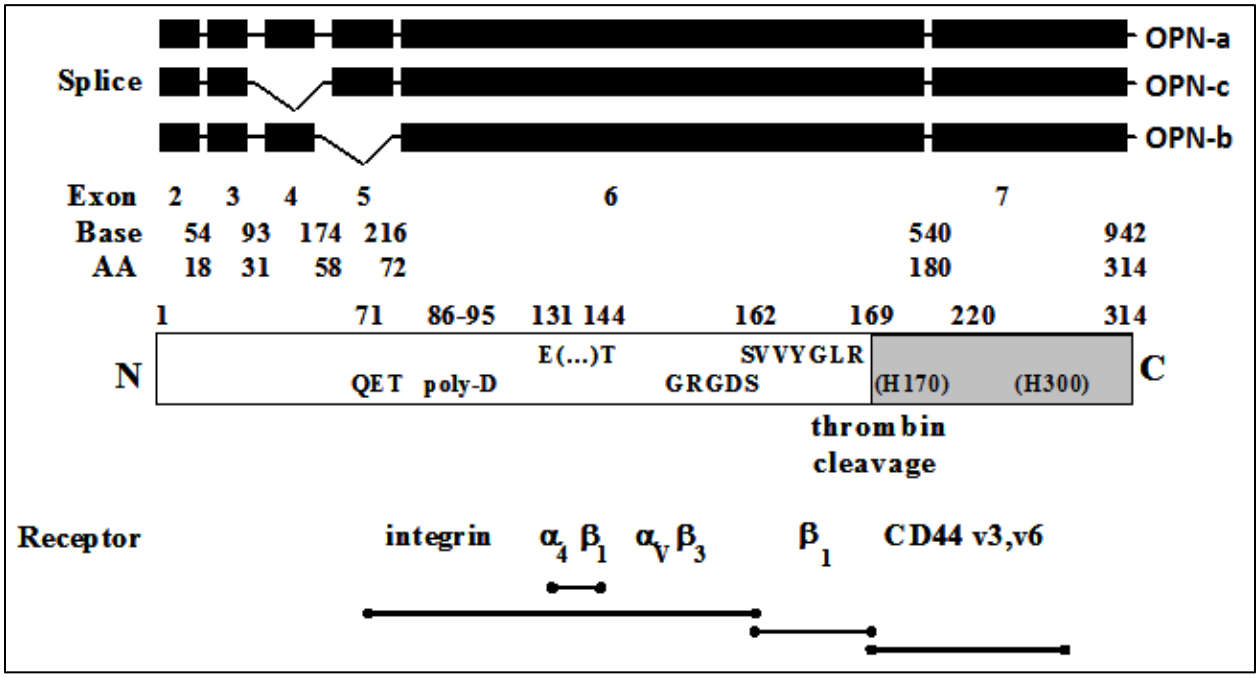


**Figure 1.5 Osteopontin Gene Product.** SPP1 gene has six translated exons. The two main domains of OPN are separated by a thrombin cleavage site. OPN undergoes splicing to form three different products: OPNa (the full-length form of OPN), OPNb (lacks exon 5) and OPNc (lacks exon 4) (He et al., 2006).

### 1.4.2. Alternative Splicing

The osteopontin gene product is subject to alternative splicing. OPNa is the full length form of OPN, OPNb lacks exon 5 and the shortest form of OPN, OPNc, lacks exon 4 (He et al., 2006). OPNa is expressed in both transformed as well as non-transformed cells. On the other hand, OPNc is selectively expressed by transformed cells alone and its levels are directly proportional to tumor grade. OPNc is always known to be expressed along with OPNa. While OPNc is a stronger promoter of anchorage-independent growth of cancer cells over OPNa, OPNa may have both pro-adhesive or pro-metastatic effects, depending on whether it is cross-linked or soluble (Weber, 2008). Both OPNb and OPNc splice forms are known to promote various aspects of tumor progression including cell proliferation, invasion, migration, EMT and expressions of MMP-2, MMP-9 & VEGF mainly via PI3K signaling (Mello et al., 2014; Tilli et al., 2012).

OPNa and OPNc may synergize to support anchorage-independent survival of transformed cells. There is loss of ATP in cancer cells under deadherent conditions, which needs to be overcome for cell survival in order to promote metastasis (Schafer et al., 2009). OPNa increases the cellular glucose levels in deadherent conditions, thus providing a mechanism for ATP generation (Shi et al., 2014a) and OPNc supports cell survival by enhancing the energy metabolism in cancer cells via redox signaling (Shi et al., 2014b). OPNc induces oxidoreductase through mitochondrial respiratory chain or hexose monophosphate shunt that leads to a generation of reactive oxygen species, which activates the tyrosine kinase SRC to promote the cell survival signals.

### 1.4.3. Aberrant Expression

Aberrant expression of genes in cancer is a common event that may lead to either upregulation of gene expression and their corresponding proteins that promote tumor progression or downregulation of the activity of tumor suppressor genes. Various growth factors affect the

expression levels of OPN. The promoter region of OPN contains binding sites for transcription factors acting downstream of these growth factors. Thus, signaling through certain receptors is able to induce higher or lower OPN expression, hence, affecting tumor progression. For instance, Smad3 (a downstream target of transforming growth factor β) and Hoxa-9 have binding sites in OPN promoter (Noda et al., 1988; Shi et al., 2001). Signaling via EGF (Atkins et al., 1997; Chackalaparampil et al., 1996), FGF (Iseki et al., 1997; Iseki et al., 1999; Tang et al., 1996), glucocorticoids (Goppelt-Struebe et al., 2000; Singh et al., 1995), estrogen (Vanacker et al., 1998; Zirngibl et al., 2008) & progesterone (Omigbodun et al., 1997) response elements, etc. all affect OPN gene expression. Table 1.1 describes the various response elements in the OPN promoter and its corresponding transcription factors that are known to bind OPN promoter and affect its protein levels.

**Table 1.1 Osteopontin Promoter.** This table depicts the various recognition elements and binding sites for transcription factors in the promoter region of osteopontin gene. (Weber, 2001)

| Base | Recognition site | Transcription factors |
|---|---|---|
| **First intron (enhancer)** | palindromic recognition element | Oct-4 |
| **(adjacent to Oct-4 site)** | | Sox-2 repressor |
| **Downstream of transcription initiation** | inverted phorbol ester responsive site | AP-1 |
| **70 to 82** | | BPV-E2 |
| **−27 to −21** | TATA box | |
| **−39 to −34** | | Ets-1, in cooperation with PEBP2αA/CBFA1 |
| **−45 to −22** | RE-1b | Oct-1, Oct-2 |
| **−53 to −49** | v-Src response element | CBF-like factor |
| **−86 to −55** | RE-1a | Myc, Sp1, glucocorticosteroid receptor, E-box binding factor |
| **−120 to −115** | Pu box | Pu.1 |
| **−123 to +66** | | USF1 |
| **−180 to −229** | | Smad3 |
| **(adjacent to −180 to −229)** | | Smad4 competing with Hoxa-9 repressor |
| **−270 to 259** | | PEA-3, IE1.2 |
| **−312 to −305** | | PEA-1 |
| **−413 to −405** | | PEA3/EBP20 |
| **−492 to −481** | | AP-2 |
| **−500 to −494** | | AP-4 |
| **−718 to −714** | | AP-1, FSE2.1 |
| **−725 to −712** | Ras-activated enhancer sequence | MATF |
| **−758 to −741** | vitamin D response element | VDR/RXR |
| **Not mapped** | progesterone regulatory element | |
| **Not mapped** | SF-1 response element | ERRα, suppression by estrogen or PTH |
| **Not mapped** | | Ets-2 |

### *1.5. Tissue Factor*

Tissue factor, or thromboplastin, is a 47 kDa transmembrane glycoprotein. Its role in the extrinsic pathway of clotting cascade, along with its role in the smooth operation of the intrinsic pathway, has been well established (Figure 1.6).

Tissue factor (TF) has six translated exons and the full-length (flTF) form of the protein consists of 263 amino acids. Exon 1 consists of the N-terminal signal sequence, exons 2-5 span amino acid sequence from 1 to 219 and form the extracellular domain, exon 6 forms the transmembrane region (amino acids 220-244), and the cytoplasmic tail consists of amino acids 245-263 (van den Berg and Versteeg, 2010).

Expression of flTF is confined to subendothelial layer, but upon injury it becomes exposed to factor VII (FVII) in the blood-stream to form a proteolytic complex flTF:FVIIa, which subsequently activates factor X (FX) and then prothrombin to finally form the fibrin clot. TF is also found circulating in the blood either on the surface microparticles or activated monocytes in response to an infection or injury (Aras et al., 2004; Giesen et al., 1999)..

Normally, TF in blood vessels is primarily located in adventitial cells, variably present in cells composing the medial layer, and is absent in endothelium and peripheral blood cells (Drake et al., 1989). Tissue Factor is expressed in response to injury, as well as to a number of different extracellular stimuli, including LPS, TNF-α, biogenic amines, interleukin-1β, VEGF, thrombin, oxidized LDL, plasmin, angiotensin II and hypoxia (Cui et al., 2003; Rong et al., 2006; Steffel et al., 2006). In addition to its role in the coagulation cascade, it has been shown to participate in a variety of physiological processes distinct from hemostasis, including embryogenesis,

inflammation, cellular signaling, cell migration, tumor growth, metastasis and angiogenesis (Belting et al., 2005; Parry and Mackman, 2000).

Tissue factor has been associated with pro-coagulant activity in many tumors (Kasthuri et al., 2009; Rao, 1992). Some studies have also correlated up-regulation of tissue factor with tumorigenesis. TF expression is higher in more malignant gliomas (Hamada et al., 1996) and metastatic breast carcinomas (Zhou et al., 1998) and colorectal cancers (Seto et al., 2000). TF mediates intracellular signaling via both clotting-dependent and clotting-independent pathways. Clotting-dependent pathways require TF-dependent thrombin generation and subsequent deposition of cross-linked fibrin and platelet activation, leading to tumor angiogenesis. Clotting-independent pathways may be mediated either by TF-FVIIa binding, the ternary TF-FVIIa-thrombin complex or by phosphorylation of TF cytoplasmic domain (figure 1.6).

Tumor hypoxia has been shown to up-regulate, egr-1 dependent, tissue factor expression in glioblastoma multiforme (Rong et al., 2006). However, the role of tissue factor in tumor progression in hypoxic, advanced stage tumor microenvironment remains to be investigated.

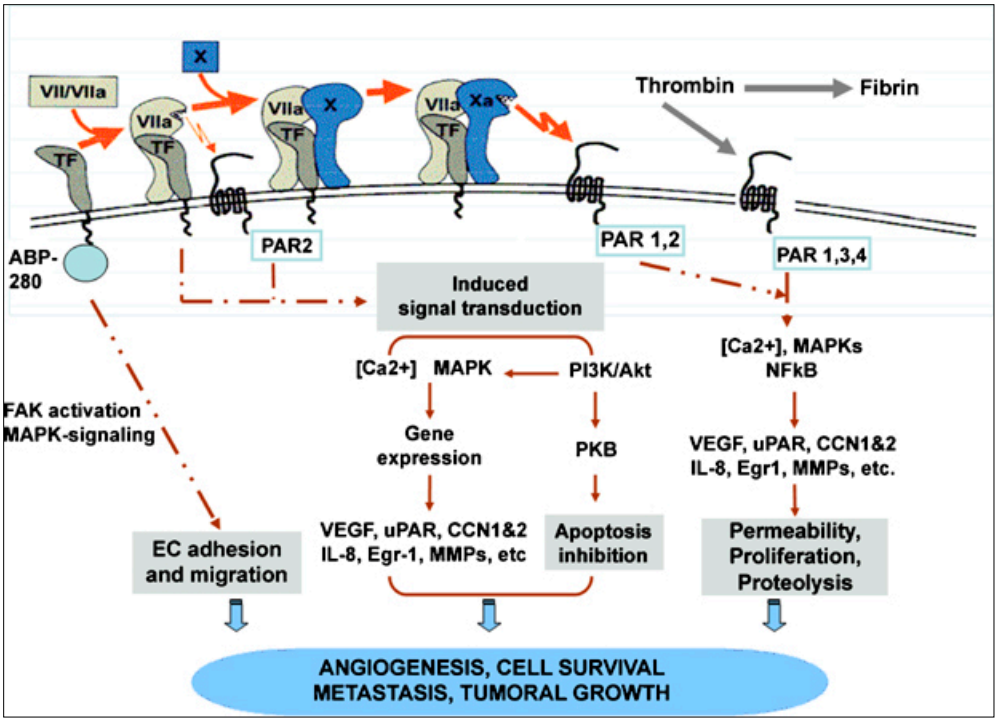


**Figure 1.6 Tissue Factor (TF) Signaling.** TF mediated intracellular signaling pathways involved in angiogenesis, cell survival, metastasis and tumor growth. *(López-Pedrera et al., 2006)*

### 1.5.1. flTF in Coagulation

flTF regulates the rate limiting step in the extrinsic pathway of the coagulation cascade (figure 1.7). As TF is exposed to the bloodstream upon injury, it binds to the circulating FVII, to induce a cascade of serine protease activations, converting: TF/FVII to TF/FVIIa, FX to FXa, prothrombin to thrombin, fibrinogen to fibrin, fibrin deposition activates the platelets to finally form the fibrin plug.

Tissue factor protease inhibitor (TFPI) is another serine protease that acts as a negative regulator of the effects of TF in the blood coagulation cascade. TFPI either binds to the TF/FVIIa complex to inhibit its effects or it binds to FXa to mediate its negative regulation. Therefore, FXa regulates its own negative feedback regulation.

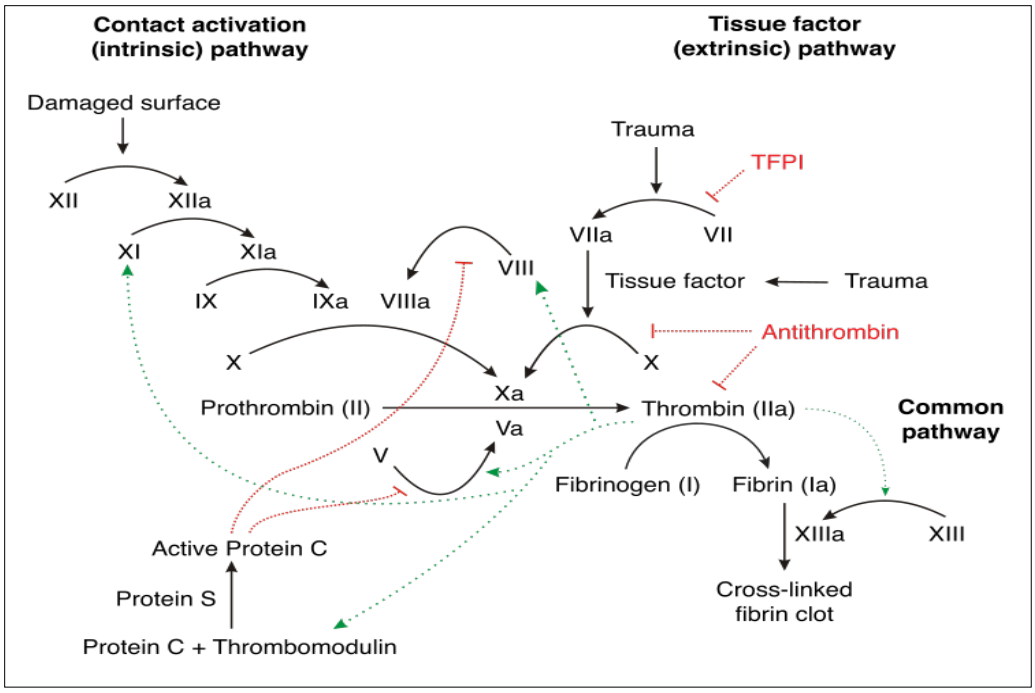


**Figure 1.7 The Coagulation Cascade.** Tissue factor is involved in the rate-limiting step of the extrinsic coagulation pathway and is responsible for the conversion of factor VII to factor VIIa. (wikipedia.org/wiki/Tissue_factor)

### 1.5.2. flTF in Cancer

flTF or flTF/FVIIa have been associated with various cancerous complications including tumor growth, progression, thrombosis, angiogenesis, etc (Rak et al., 2006; Rickles et al., 2003; Ruf and Mueller, 2006; Yu et al., 2005). flTF in combination with FVIIa regulates tumor angiogenesis via PAR-2. TF – FVIIa – PAR-2 regulates the expression of VEGF, IL-8, metalloproteinases and CXCL-1 (Versteeg et al., 2008). Depending on the oxidation state of flTF, it is able to regulate various cancer-associated functions. The reduced state of flTF in association with PAR-2 is involved in tumor angiogenesis. On the other hand, the oxidized state of flTF promotes metastasis via PAR-1 activation and the coagulation cascade (Ahamed et al., 2006). flTF is also known to interact with integrins (α6β1, α3β1) to influence angiogenic signaling (Dorfleutner et al., 2004; Versteeg et al., 2008).

### 1.5.3. Alternative Splicing

Alternative splicing is a mechanism whereby a single gene encodes for multiple proteins. The full length form of tissue factor is a cell surface glycoprotein. The gene has six translated exons. Upon splicing, exon 5 gets deleted and exon 4 is directly joined to exon 6, causing a frameshift mutation in the primary transcript to form an alternatively spliced variant of tissue factor (asTF). This form consists of 206 amino acids. The amino acid sequence from 1-166 is similar to that of the full length form, whereas 167-206 amino acids are different leading to a variant cytoplasmic domain. In contrast to the full length form, asTF lacks its transmembrane domain and is thus soluble. The various features of the full length and alternatively spliced tissue factor are compared in figure 1.8.

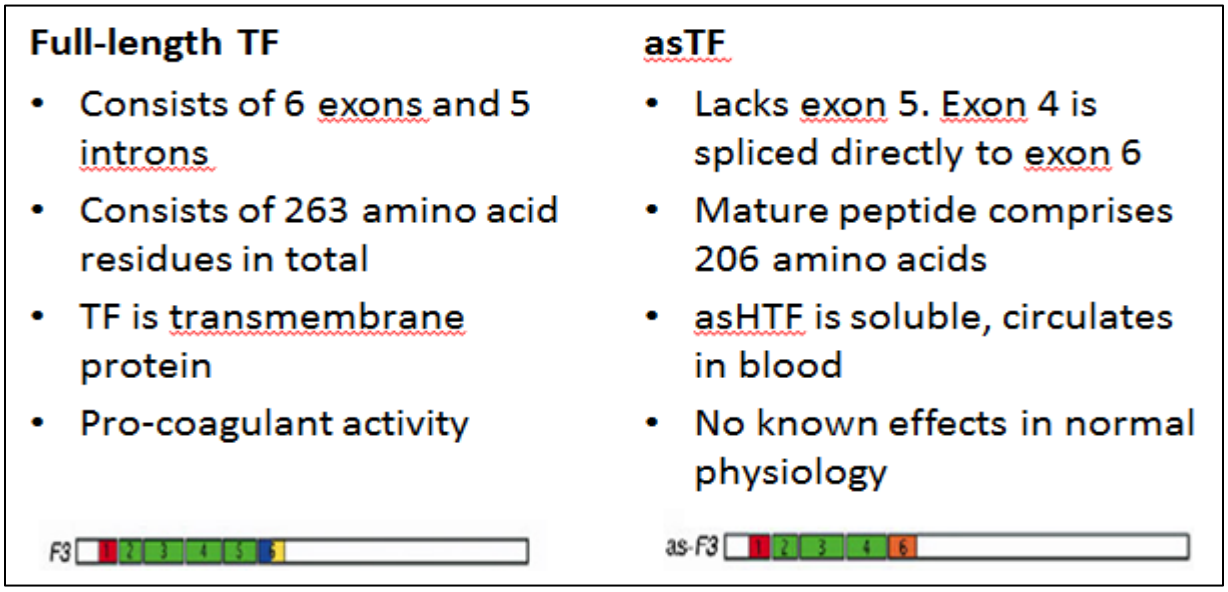


**Figure 1.8 Alternatively Spliced form of Tissue Factor (asTF).** This figure compares the structural and functional features of full-length TF (flTF) and the alternatively spliced variant of TF (asTF).

Pre-mRNA splicing depends on the phosphorylation status of serine/arginine (SR) proteins by their binding to exonic splicing enhancer (ESE) motifs. Exon 5 of TF gene consists of ESE motifs that bind to SF2/ASF, SRp55, SC35 and SRp40. Selective mutagenesis in SF2/ASF and

SRp55 weakens SP protein - pre-mRNA binding, whereas SC35 and SRp40 compete with SF2/ASF and SRp55 binding to promote exon exclusion, hence, regulating pre-mRNA splicing of TF in monocytes (Chandradas et al., 2010; Tardos et al., 2008). In endothelial cells TNF-α stimulation regulates TF splicing via SRp75, SRp55 and SF2/ASF (Szotowski et al., 2005).

### 1.5.4. asTF and Coagulation

Despite the presence of the functional lysine doublet (AA 165-166), the pro-coagulant activity of asTF has always been subject to debate. Since, a major part of the extracellular domain of asTF is replaced with a very different C-terminus, there have been controversial views on its coagulant activity. In the presence of phospholipids, asTF has been shown to propagate formation of in vivo thrombi (Bogdanov et al., 2003). After stimulation with IL-6 and TNF-α and in the presence of phospholipids, asTF generates FXa (Szotowski et al., 2005). In contrast, some studies did not find any evidence of pro-coagulant activity by asTF (Böing et al., 2009; Censarek et al., 2007).

### 1.5.5. asTF and Cancer

The importance of TF:fVIIa in cancer has been well justified in literature. Since the identification of asTF, its role in cancer remains to be elucidated. Majority of pancreatic cancer cell lines and pancreatic cancer patients have shown asTF expression (Haas et al., 2006). Higher asTF expression correlates to higher grade and poor clinical outcome in NSCLC patients (Goldin-Lang et al., 2008; Rollin et al., 2010). asTF has also been found to have a differential expression in patients with cervical cancer (van den Berg et al., 2009). In contrast, flTF expression, rather than asTF, correlates better with cancer progression in patients with colorectal (Yu and Rak, 2004) and esophageal (Ribeiro et al., 2009) cancers.

The mechanism of action of asTF in various aspects of cancer progression still remains to be determined. Unlike flTF, asTF does not act via fVIIa and PAR2, but mediates its various effects via integrin binding. asTF mediates endothelial cell migration via $\alpha_v\beta_3$ and capillary formation via its interaction with $\alpha_6\beta_1$. Binding of asTF with integrins leads to phosphorylation of kinases including FAK, PI3 kinase, p38 MAPK, p44/42 MAPK and Akt. PI3 kinase, p38 MAPK and Akt are involved in endothelial cell migration, whereas PI3K and p44/42 MAPK are crucial for capillary formation.

Even though asTF expression has been seen in various cancer types, the correlation of its expression levels with clinical outcome and various tumor characteristics needs to be properly elucidated.

# Chapter 2
# Research Significance & Objectives

# 2. Research Significance & Objectives

## *2.1. Significance*

The aim of this study was to characterize the two types of genetic alterations in metastatic genes and correlate them with aggressiveness of solid tumors. The study is divided into two parts, based on the type of genetic alteration of interest.

The first part of this project has been devoted to aberrant expression of metastatic genes. This form of genetic alteration was studied in the context of OPN. OPN splice variants have been identified and studied in breast cancer, but the role of OPN expression in breast cancer aggressiveness has not been studied previously.

The second part of this project evaluates the impact of an alternatively spliced variant of another gene, called tissue factor (TF) on pancreatic cancer growth and progression. Since, the expression of asTF is profound in the advanced stages of cancer, the pancreatic cancer cell line was over-expressed for asTF and the experiments were performed under hypoxic, low glucose and acidic conditions, closely resembling those around the tumor physiologically.

## *2.2. Hypothesis*

I. High expression polymorphisms in the osteopontin promoter lead to an elevated risk for tumor aggressiveness in breast cancer over coding-region polymorphisms in the SPP1 gene

II. Expression of alternatively spliced variant of Tissue Factor up-regulates certain hypoxia-inducible genes and thus, plays an important role in the progression of pancreatic cancer in an advanced-stage Tumor Environment.

## 2.3.*Specific Aims*

### 2.3.1. **Specific Aim 1:** An Osteopontin Promoter Polymorphism is Associated with Aggressiveness in Breast Cancer

*Aim 1(a):* To study the association of SPP1 promoter polymorphic sites with breast cancer aggressiveness in comparison to coding region polymorphisms of SPP1 gene.

*Aim 1(b):* To analyze the correlation of promoter polymorphisms with the levels of OPN expression

### 2.3.2. **Specific Aim 2:** The role of alternatively spliced Tissue Factor in Pancreatic Cancer

*Aim 2(a):* Analyze the role of alternatively spliced Tissue Factor (asTF) in tumor growth and progression under advanced stage tumor microenvironment

*Aim 2(b):* Evaluate the intracellular signaling mechanisms that are responsible for tumor progression under hypoxia

*Aim 2(c):* Test mechanisms in *in vivo* systems

# Chapter 3

# An Osteopontin Promoter Polymorphism is Associated with Aggressiveness in Breast Cancer

# 3. Osteopontin Promoter Polymorphisms are associated with aggressiveness in Breast Cancer

### *3.1. Introduction*

Cancer initiation commonly occurs when the coding region of a proto-oncogene is mutated to induce a gain of function of the resulting gene product, such that it is excessively active, or when the coding region of a tumor suppressor gene suffers a mutation that inactivates the resulting gene product. In either case, the pathophysiologic consequence is excessive cell cycle progression or defective programmed cell death. Cancer progression and metastasis are also genetically programmed. However, whereas mutations in the coding regions of critical genes underlie early transformation, metastasis gene products are typically not mutated in cancer. We have previously shown that aberrant expression or splicing of metastasis genes underlie tumor progression (Ashkar et al., 2000; Weber, 2008; Weber and Ashkar, 2000b).

Osteopontin is a metastasis gene that contributes to the progression of over 30 forms of cancer (Weber, 2011; Weber et al., 2010, 2011). Aberrant splicing of osteopontin in cancers has been accounted for by our identification of the variant form osteopontin-c, which is selectively expressed in cancer cells but is absent from non-transformed cells (Jennifer et al., 2009; Mirza et al., 2008; Tilli et al., 2011). Osteopontin-c supports anchorage-independent survival and expansion, which is an essential component of tumor dissemination (He et al., 2006).

Although osteopontin (encoded by the gene spp1) has been known to be produced at elevated levels by cancer cells (Senger et al., 1983), the molecular underpinning for its aberrant expression in cancer is incompletely accounted for. Osteopontin may be induced as a downstream signal transduction target of proto-oncogenic growth factors (Noda et al., 1988) or

secondary to gain-of-function events in transforming signaling pathways (Chambers et al., 1992; Whalen et al., 2008; Zhang et al., 2003). In either case, the binding of cognate transcription factors to spp1 promoter regions is causative for the upregulated expression. This opens the possibility that mutations or polymorphisms in the promoter of the spp1 gene (Figure 1) may predispose to various levels of expression after transformation, and hence to various levels of tumor aggressiveness. Here we investigate this hypothesis for breast cancer.


(A)
-2053A/T
-1983C/T rs114708554
-1968T/C rs192504364
-1955A/C rs139281215
1896C/T rs180827899
1893 ins A
-1776T/C ss32469552, rs29001511
-1748A/G rs2728127
-1685A/G
-1593A/C rs114427432
-1441C/T rs186128765
-1461C/T rs190066547
-1450A/C rs182762181
-1332A/G
-1100A/T rs59641329
-1071A/C rs142608941
-1041G/T rs3891314
-956C/T rs190538514
-670A/G rs183186314
-655G/T
-616G/T rs2853744
-593T/A
-567A/G rs188770751
-458A/G rs74705290
-443T/C rs11730582
-430G/T rs41351345
-339A/G ss457263976rs193258428
-319C/G rs2853745
-302A/C rs117291487
-281C/T rs75730736
-261A/G rs149628949
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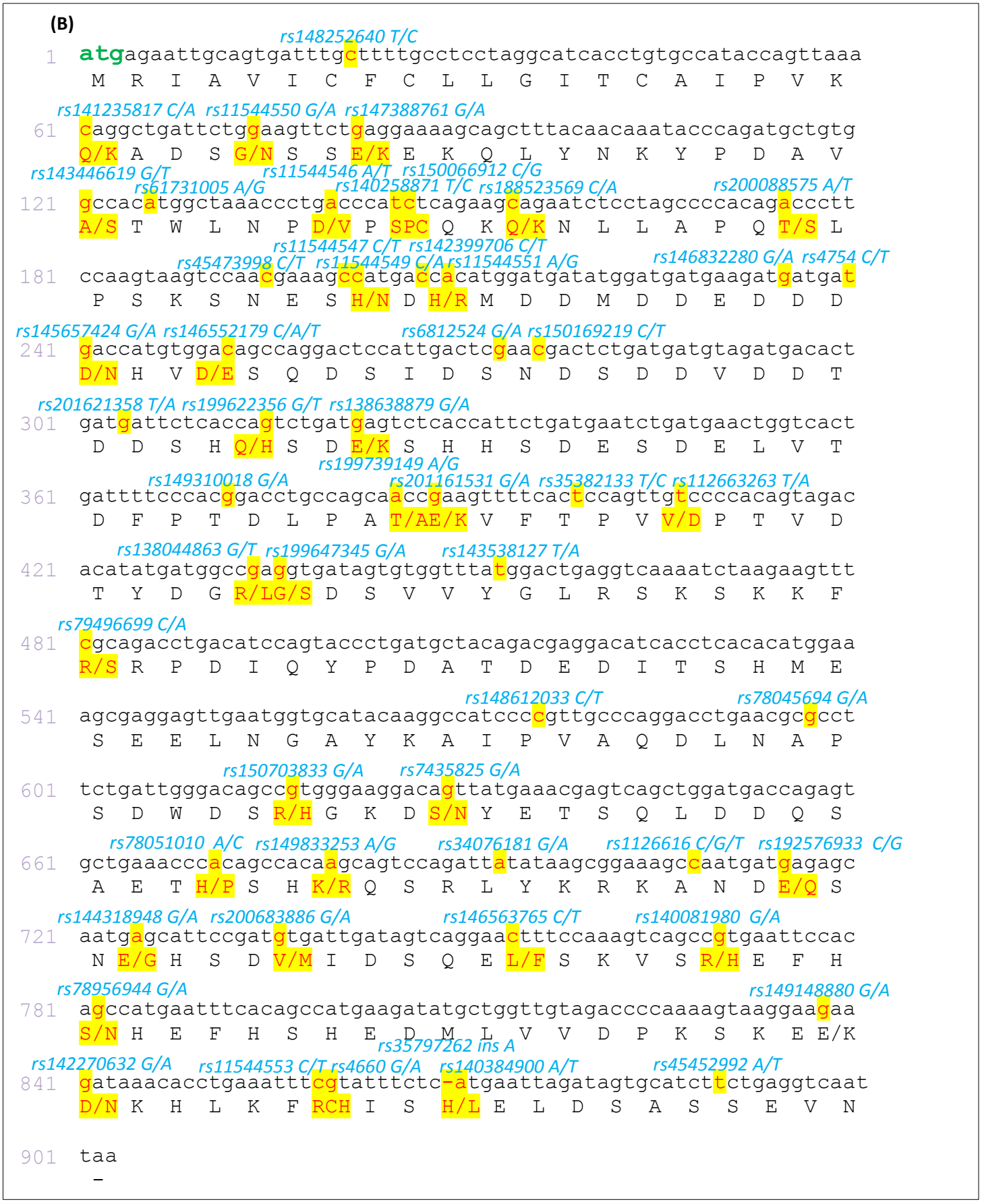
(B)
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rs141235817 C/A rs11544550 G/A rs147388761 G/A
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Q/K A D S G/N S S E/K E K Q L Y N K Y P D A V
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rs61731005 A/G rs140258871 T/C rs188523569 C/A rs200088575 A/T
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A/S T W L N P D/V P SPC Q K Q/K N L L A P Q T/S L
rs11544547 C/T rs142399706 C/T
rs45473998 C/T rs11544549 C/A rs11544551 A/G rs146832280 G/A rs4754 C/T
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P S K S N E S H/N D H/R M D D M D D E D D D
rs145657424 G/A rs146552179 C/A/T rs6812524 G/A rs150169219 C/T
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D/N H V D/E S Q D S I D S N D S D D V D D T
rs201621358 T/A rs199622356 G/T rs138638879 G/A
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D D S H Q/H S D E/K S H H S D E S D E L V T
rs199739149 A/G
rs149310018 G/A rs201161531 G/A rs35382133 T/C rs112663263 T/A
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T Y D G R/LG/S D S V V Y G L R S K S K K F
rs79496699 C/A
481 cgcagacctgacatccagtaccctgatgctacagacgaggacatcacctcacacatggaa
R/S R P D I Q Y P D A T D E D I T S H M E
rs148612033 C/T rs78045694 G/A
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S E E L N G A Y K A I P V A Q D L N A P
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S D W D S R/H G K D S/N Y E T S Q L D D Q S
rs78051010 A/C rs149833253 A/G rs34076181 G/A rs1126616 C/G/T rs192576933 C/G
661 gctgaaacccacagccacagcagtccagattatataagcggaaagccaatgatgagagc
A E T H/P S H K/R Q S R L Y K R K A N D E/Q S
rs144318948 G/A rs200683886 G/A rs146563765 C/T rs140081980 G/A
721 aatgagcattccgatgtgattgatagtcaggaactttccaaagtcagccgtgaattccac
N E/G H S D V/M I D S Q E L/F S K V S R/H E F H
rs78956944 G/A rs149148880 G/A
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S/N H E F H S H E D M L V V D P K S K E E/K
rs35797262 ins A
rs142270632 G/A rs11544553 C/T rs4660 G/A rs140384900 A/T rs45452992 A/T
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D/N K H L K F RCH I S H/L E L D S A S S E V N
901 taa
-

**Figure 3.1 Spp1 polymorphisms.** (A) Promoter. The sequence is derived from NW_001838915.1 (whole genome shotgun sequence) and NT_016354.19 (genomic contig) (the 160 bases proximal to the transcription start site are also confirmed by GenBank nos. NM_001040058.1, NM_001040060 and NM_000582; of note, the GenBank sequences S78410.1 and D14813.1 contain a 60 nucleotide gap which is likely a cloning artifact). The silent exon 1 is grey. The 'A' that starts the conventional numbering of the promoter sequence is displayed in bold green font, as is the downstream ATG start site. The literature has identified 12 polymorphic sites (red letters on yellow background) in the spp1 promoter, of which 6 have rs or ss numbers (positions −66, −156, −443, −616, −1748, −1776) (22, 15, 26, 27, 16). Additional polymorphic sites have been reported, and comprise −145/−146 (28), −302 (28), −593 (29), −655 (30), −1282 (31), −1625 (31) and −2053 (15). With the exception of one insertion/deletion polymorphism at position −156, all are single nucleotide exchanges. The position and nature of the polymorphism is indicated above each site in blue. (B) Coding sequence. The polymorphisms with assigned rs numbers are identified in the NCBI SNP database. The protein sequence is shown under the nucleotide sequence, and for non-synonymous base changes the amino acid changes are depicted in red letters on yellow background. The position and nature of the polymorphism is indicated above each site in blue.

## *3.2. Methods*

### 3.2.1. Patients

There were four sources of specimens. DNA from breast cancer patients and healthy controls was obtained from the Division of Human Genetics at The Ohio State University (50 breast cancers, 50 untransformed surrounding tissues, 50 healthy breasts). From tumors previously analyzed for osteopontin RNA expression (Mirza et al., 2008), DNA was obtained by phenol/chloroform extraction (23 breast cancers, 11 surrounding tissues, 15 healthy breasts). DNA from breast cancers and surrounding tissues was purchased from Bioserve (86 tumors and 50 untransformed surrounding tissues) and from Origene (82 tumors). The total number of samples was 241 breast cancers, 111 surrounding tissues, 65 healthy breast specimens.

### 3.2.2. DNA Genotyping

Genotyping was done using ABI PRISM 7900HT Sequence Detection System after performing the polymerase chain reaction on the DNA samples. The PCR was done as directed by the ABI protocol for the TaqMan SNP Genotyping assays using TaqMan Universal PCR master mix, primer and TaqMan Probe (VIC/FAM) dye mix, and 5 ng/µl genomic DNA sample. The total

reaction volume was 5 µl. Then, post-PCR plate reads were performed by the Sequence Detection System instrumentation to identify the distinct alleles according to their fluorescent signals. One probe set tested the spp1 polymorphic promoter sites -66 (rs28357094), -443 (rs11730582), -1748 (rs2728127) and -1776 (rs29001511). We also set out to investigate non-synonymous DNA sequence variations in the coding region. The available probes for this comprised the positions 305 (rs11544546), 367 (rs11544549), 794 (rs7435825) and 1025 (rs4660) and were all included in this study.

### 3.2.3. RNA and real-time RT-PCR

Specimens of human breast tumors, non-transformed surrounding tissue, as well as healthy breast tissue (obtained from reduction mammoplasties) were provided by the tissue procurement facility of the University of Cincinnati Medical Center/Children's Hospital (Mirza et al., 2008). Total RNA was extracted from specimens using TRIZOL Reagent (Invitrogen). Total RNA was used for cDNA synthesis by reverse transcription with Superscript II (Invitrogen) according to the manufacturer's protocol in a total volume of 20 µl.

All PCR reactions were performed on a Cepheid (Sunnyvale, CA) Smart Cycler using SYBR Green detection format. 0.5 µl of cDNA was added to each PCR reaction in a total volume of 25 µl using the standard Invitrogen PCR buffer system with optimized concentrations of $MgCl_2$. For each experiment a no-template reaction and cDNA from the reference cell line MDA-MB-435 were included as negative and positive controls. The conditions for PCR were 94$^{o}$C denaturation for 120 s followed by 40 cycles of: 94$^{o}$C melting for 15 s, a primer set specific annealing temperature for 30 s (Mirza et al. 2008), extension at 72$^{o}$C for 30 s, and ending with a melting curve program (60-95$^{o}$C with a heating rate of 0.2$^{o}$C and a continuous fluorescence measurement), and finally a cooling step to 4$^{o}$C. Product purity, product size, and absence of

primer dimers were confirmed by DNA melting curve analysis. Melt curves yielded a single sharp peak for all template reactions, and a minimal melt peak (resulting from primer dimers) or no melt peaks for the no-template control reactions.

#### 3.2.4. Statistics

We performed the Hardy-Weinberg exact test to analyze deviations from equilibrium and association analysis to evaluate genetic effects on phenotype using the statistical software R (www.R-project.org). Single nucleotide polymorphisms (SNPs) whose genotype frequencies departed from Hardy-Weinberg equilibrium at $p<0.01$ were filtered out. Thus, we evaluated associations among the three promoter SNPs rs11730582, rs2728127 and rs29001511 in the promoter region with various breast cancer characteristics. These statistical evaluations were carried out using multivariate logistic regression under an additive genetic model by $\chi^2$ test. The accepted significance level for association analysis was 0.1.

The case-control haplotype analysis was performed using Haploview v4.2 (http://www.broad.mit.edu/mpg/haploview). Similar to the association analysis, the three SNPs: rs11730582, rs2728127 and rs29001511 in the promoter region, were used to generate haplotype frequencies, as the genotype data for these SNPs had a p-value above the cut-off value of 0.01.

### *3.3. Results*

#### 3.3.1. Patient Demographics

This study comprised 241 breast cancer specimens, for 111 of which DNA from normal surrounding tissue was available, and 65 healthy breast samples. The cohort consisted of all women. In all groups, the mean age was close to 50 years. The demographic and cancer characteristics are specified in Table 3.1.

**Table 3.1 Patient demographics.** ER, estrogen receptor; PR, progesterone receptor; na, not applicable.

| (Actual no. of samples) | | | breast cancer (241) | normal surrounding (111) | normal breasts (71) |
|---|---|---|---|---|---|
| | | | | | |
| ductal | | | 212 | 98 | 0 |
| lobular | | | 13 | 7 | 0 |
| mucinous | | | 2 | 2 | 0 |
| papillary | | | 0 | 0 | 0 |
| | | | | | |
| age (mean ± sem) | | | 52.22± 0.79 | 50.71±0.97 | 49.13±1.60 |
| | | | | | |
| race | Caucasian | | 85 | 49 | 53 |
| | Asian | | 87 | 51 | 1 |
| | Black | | 17 | 3 | 7 |
| | Hispanic | | 0 | 0 | 0 |
| | middle eastern | | 0 | 0 | 1 |
| tumor size (mean ± std. dev.) | | | 1.9±1.1 | 1.9±1.1 | n/a |
| tumor grade | 1 | | 13 | 6 | n/a |
| | 2 | | 63 | 34 | n/a |
| | 3 | | 70 | 19 | n/a |
| tumor stage | | | | | |
| | T1 | | 76 | 43 | n/a |
| | T2 | | 114 | 50 | n/a |
| | T3 | | 24 | 7 | n/a |
| | T4 | | 7 | 2 | n/a |
| | | | | | |
| | N0 | | 90 | 44 | n/a |
| | N1 | | 87 | 42 | n/a |
| | N2 | | 11 | 4 | n/a |
| | N3 | | 24 | 12 | n/a |
| | N4 | | 1 | 0 | n/a |
| | | | | | |
| | M0 | | 89 | 51 | n/a |
| | M1 | | 1 | 0 | n/a |
| | | | | | |
| tumor stage | | | | | |
| | I | | 42 | 22 | n/a |
| | II | | 130 | 60 | n/a |
| | III | | 52 | 20 | n/a |
| | IV | | 1 | 0 | n/a |
| | | | | | |
| ER | + | | 98 | 47 | n/a |
| | - | | 79 | 33 | n/a |
| PR | + | | 94 | 42 | n/a |
| | - | | 85 | 38 | n/a |
| HER2 | + | | 54 | 18 | n/a |
| | - | | 84 | 36 | n/a |

### 3.3.2. Individual Polymorphic Sites and Cancer

The polymorphic site in position -66 was not in Hardy-Weinberg equilibrium and hence was not included in further analyses. When comparing the other three promoter SNPs between cancers and healthy controls, the association analysis by $\chi^2$ test using multivariate logistic regression under an additive genetic model did not reveal significant differences between the groups for positions -443, -1748, or -1776. However, a separate analysis using the Cochran-Armitage trend test (CATT) and assuming a recessive genetic model reached accepted significance levels for all three sites, implying the possibility of a weak association of these polymorphisms with cancer. A small set of DNAs from colon samples showed a distribution of SNPs very similar to breast cancer (Figure 3.2A). For studying coding region polymorphisms that are associated with amino acid changes, four probes were available. At SNP position 305, three tumors had a deviant genotype from all other specimens. This SNP was in Hardy-Weinberg dysequlibrium. The polymorphic sites in positions 367, 794, and 1025 showed one homozygous genotype for all specimens in this study and therefore were not further analyzed (figure 3.2B).

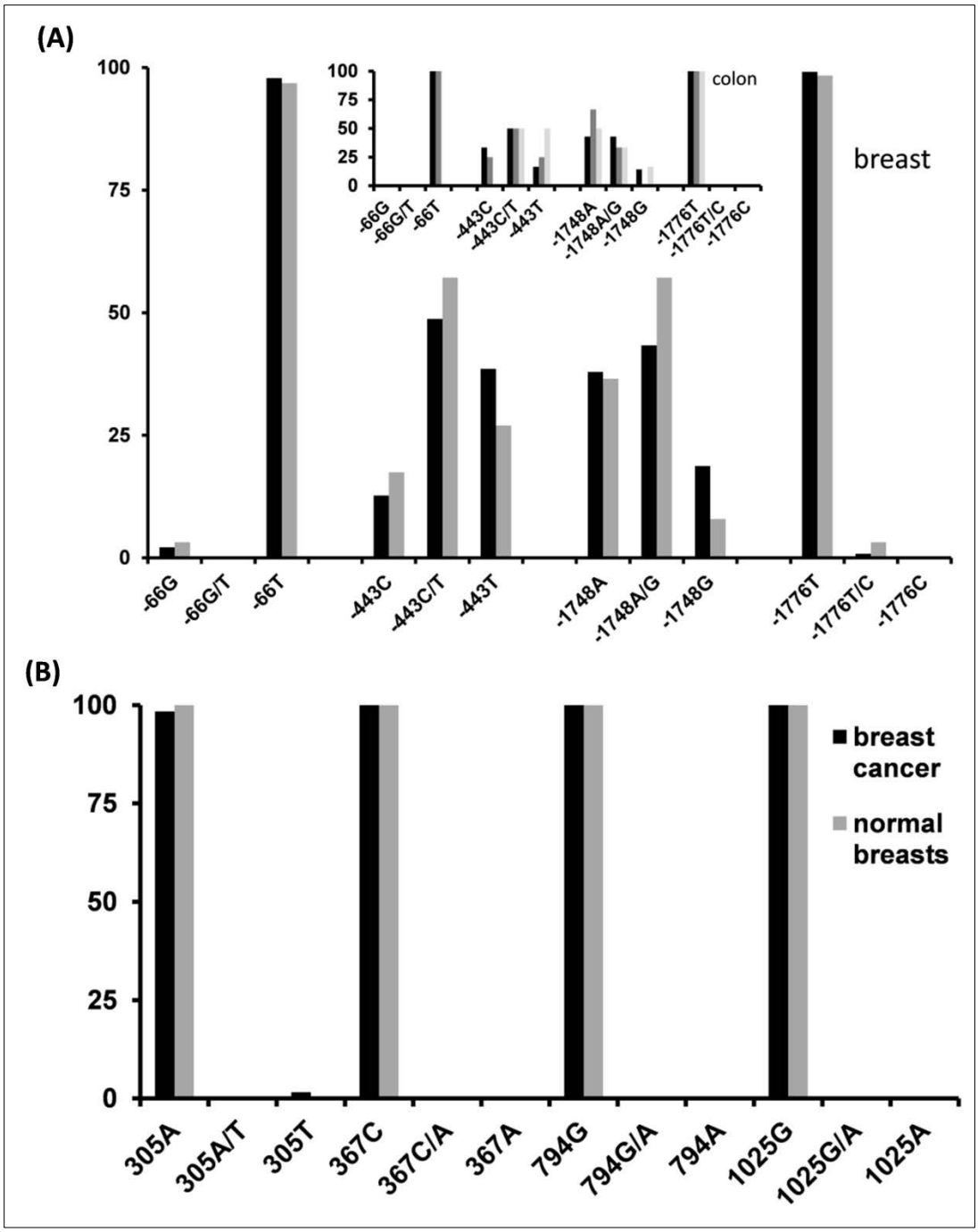



**Figure 3.2 Allelic distributions in breast cancer.** (A) Comparison of promoter polymorphisms in breast cancer (n=236) vs. healthy controls (n=63) (y-axis is percentage of the total). [Insert, for comparison, a small number of colon specimens (7 colorectal cancers-black, 4 surrounding tissues-dark gray, 5 benign growths and non-tumor disease-light gray) with a similar distribution is shown. The samples were obtained from the University of Cincinnati tissue procurement]. (B) Coding region non-synonymous polymorphic sites in breast cancer (n=210) and healthy controls (n=24).

### 3.3.3. Individual Polymorphic Sites and Clinical Measures of Cancer

Within the cancer cohort, the polymorphisms in position -443 correlated with tumor grade (after combining grades 1 plus 2 and comparing them to grade 3). The difference in position -443 between low grade and high grade cancers was confirmed by reanalysis with an allelic based test, a recessive genetic model, and an additive/multiplicative genetic model. The polymorphic site in position -1776 just barely reached significance level when grades 1 and 2 were combined and compared to grade 3. However a reanalysis of grade 1 versus grade 2 and grade 2 versus grade 3 did not indicate significant differences in genotype at this position. There was no association of the promoter SNPs with tumor stage (I-II versus III-IV) or with in situ carcinoma versus cancer (Figure 3.3). The latter results are expected as stage and early transformation are determined by the sampling time more than by tumor genetics.

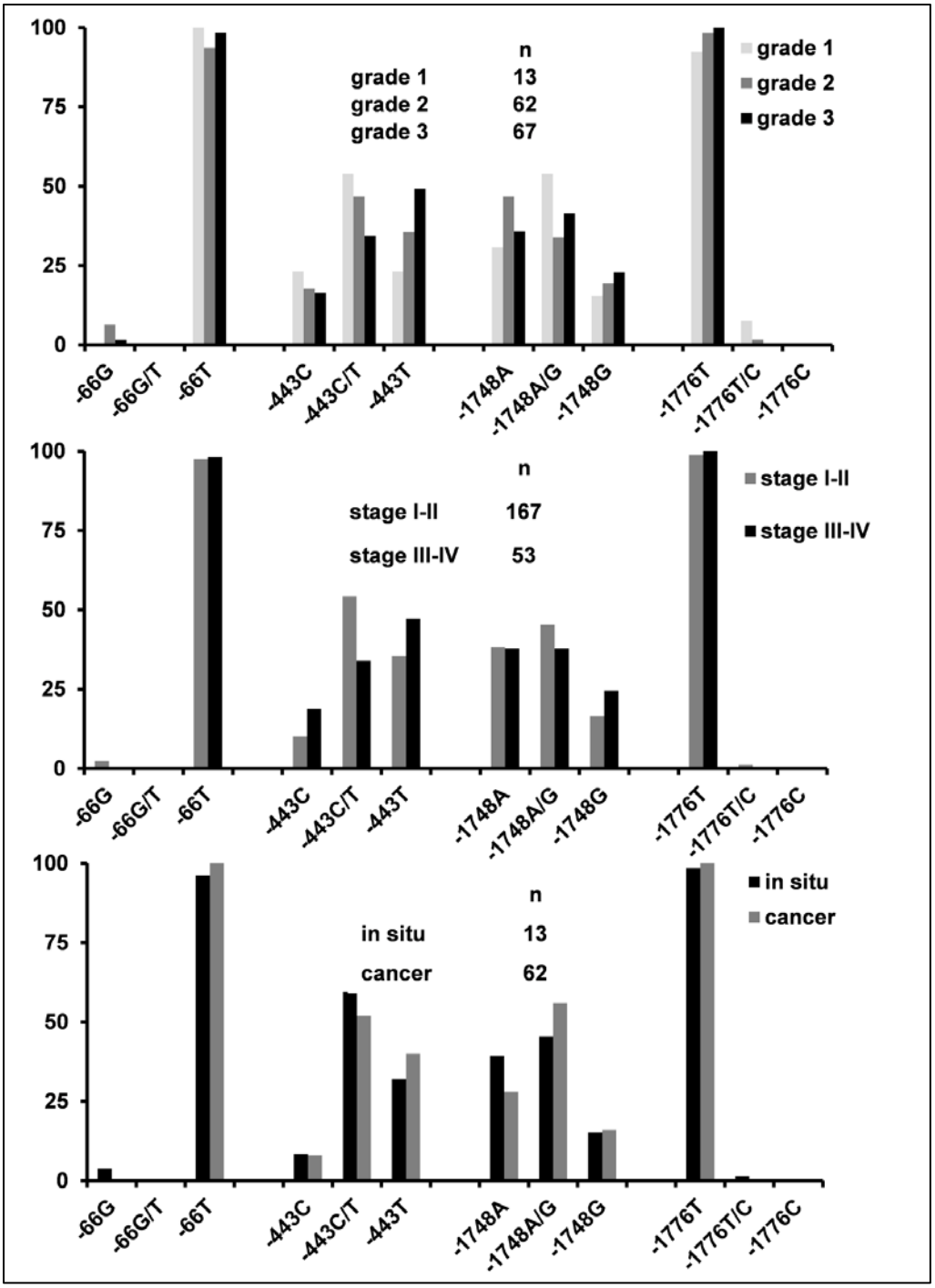



**Figure 3.3 Allelic distribution and cancer characteristics.** The top panel indicates promoter polymorphisms related to cancer grade (percent of total), the middle panel shows the relation to cancer stage, and the bottom panel reflects the comparison of in situ carcinomas vs. cancer. *Significant difference between groups at $p<0.1$;

Osteopontin expression has been associated with breast cancer progression, regardless of the histologic subtype of the cancer (Mirza et al., 2008; Weber et al., 2011). Remarkably, the polymorphic site at -443, but not -1748 or -1776, showed differences between ER positive and ER negative breast cancers and between PR positive and PR negative breast cancers, but there was no association with HER2 status. The -443 allele T was more common in the ER negative cancers and in the PR negative cancers (Figure 3.4).

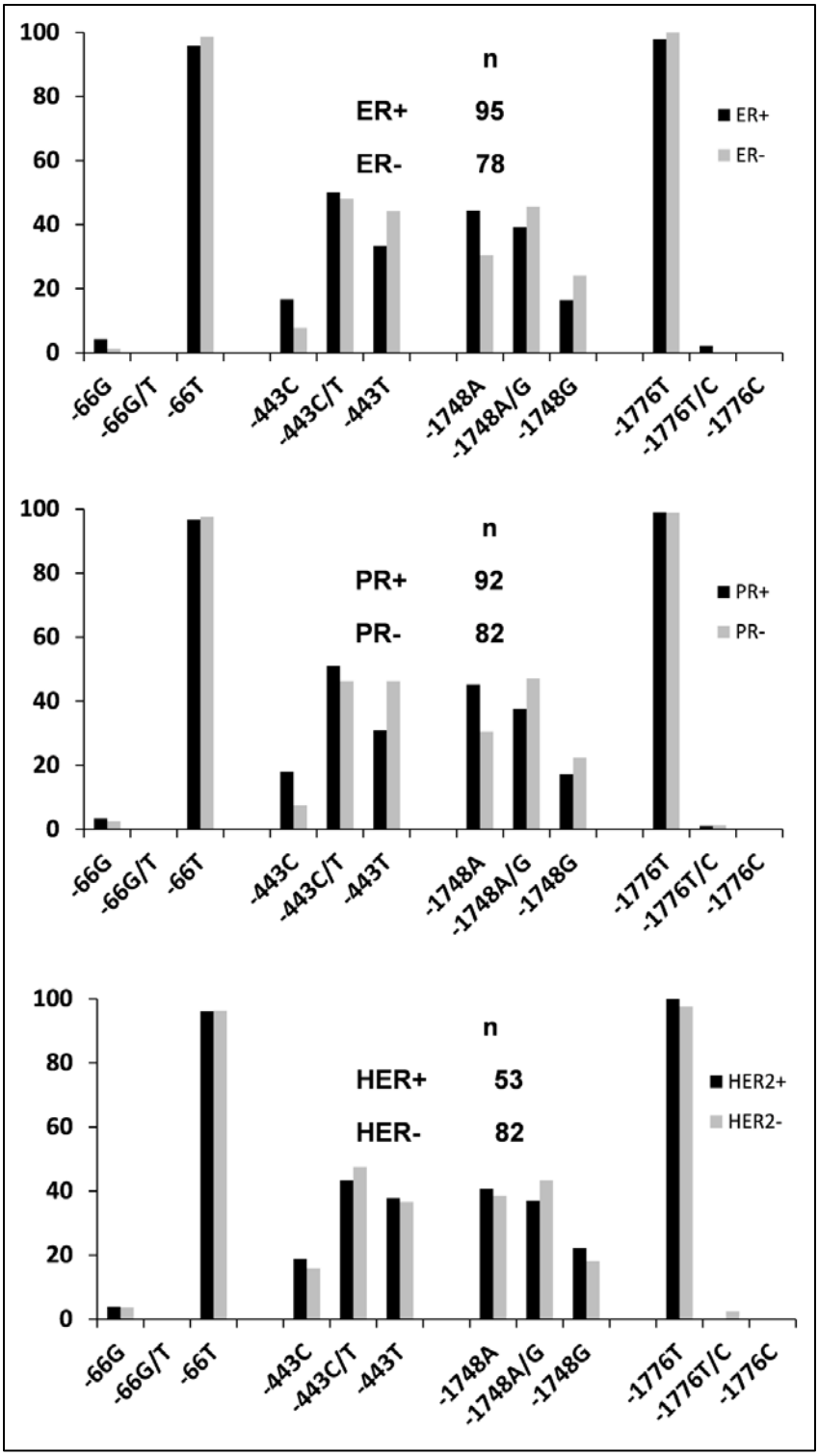



**Figure 3.4 Allelic distribution and cancer characteristics.** Osteopontin promoter polymorphisms in relation to ER expression (top panel), PR expression (middle panel), or HER2 expression (bottom panel) of the cancers. *Significant difference between groups at $p<0.1$; **significant difference between groups at $p<0.05$. ER, estrogen receptor; PR, progesterone receptor.

### 3.3.4. Somatic Mutations

For 111 cancers, we had DNA from the matching untransformed surrounding tissues available. We tested it to detect possible somatic mutations. None were found in position -66. In position -443, two tumors were heterozygous in homozygous hosts, one with the C/C genotype and the other with the T/T genotype. In position -1748, two tumors had a heterozygous genotype, changed from the host homozygous A/A. In position -1776, one tumor was heterozygous in a homozygous T/T host. These results suggest that tumors may encounter somatic mutations in the spp1 promoter that have the potential to affect expression levels. For the coding region polymorphic sites (positions 305, 367, 794, and 1025), there were no differences between tumor and untransformed surrounding tissue.

### 3.3.5. Association of Polymorphisms with Expression Levels

For a subset of samples, we had information on the expression levels of osteopontin according to real-time RT-PCR analysis from breast tissue. We asked whether the promoter haplotype correlated with expression, using 0.15 relative units as the cutoff between high and low osteopontin expression. As the allelic distribution in positions -66 and -1776 was almost homogeneous in patients as well as in normal controls, we focused on -443 and -1748. The base G in position -1748, on a homozygous or heterozygous background, was associated with higher expression levels of osteopontin RNA in the breast tissue (74% G in high expressors versus 41% G in low expressors) (Figure 3.5). Of the 6 tumors with the highest osteopontin expression (over 1.2 relative units), 4 had a G in position -1748. The polymorphic site in position -443 seemed to have a lesser effect, but the fraction of T/T was increased and the fraction of C/C was decreased in the population of high expressors compared to the low expressors (47% T/T in high expressors versus 38% T/T in low expressors).

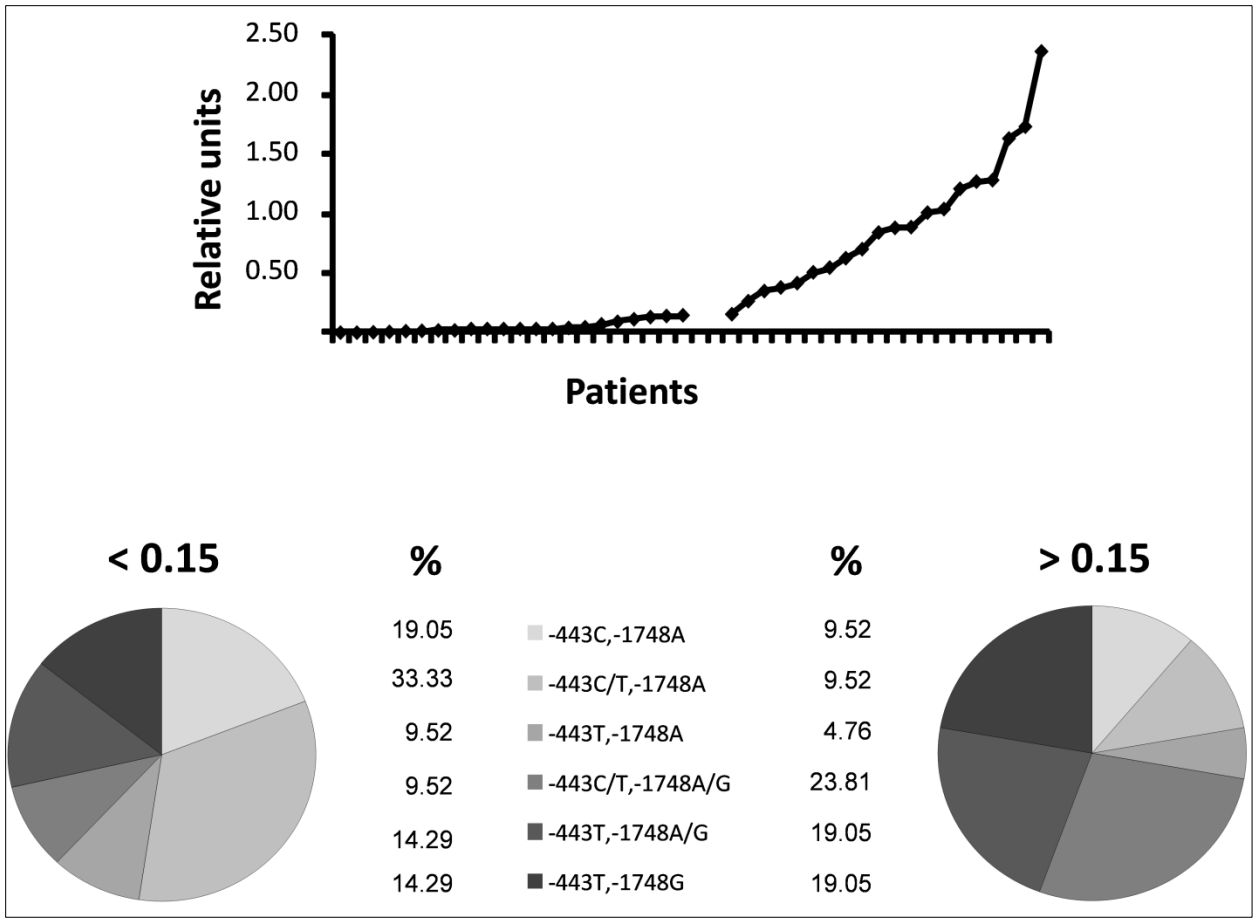



**Figure 3.5 Influence of spp1 promoter haplotype on osteopontin mRNA expression.** The samples from cancer patients and healthy controls were combined and then divided into 2 groups of low (<0.15 relative units, n=21) or high (>0.15 relative units, n=18) expression levels. Top panel: Distribution of osteopontin expression levels and population cut-off. Bottom panel: As all had the genotype T/T in positions −66 and −1776, the expression level was correlated to the haplotypes generated in positions −443 and −1748.

### 3.3.6. Haplotype Analysis

We performed a haplotype analysis for the promoter SNPs. The polymorphic site in position -66 was eliminated from this evaluation as it was not in Hardy-Weinberg equilibrium. Among the other three sites, there was an association between -443 and -1748 in the cancer patient group as well as in the healthy controls. No association was found for SNP -1776 with either of the other polymorphic sites (Figure 3.6).

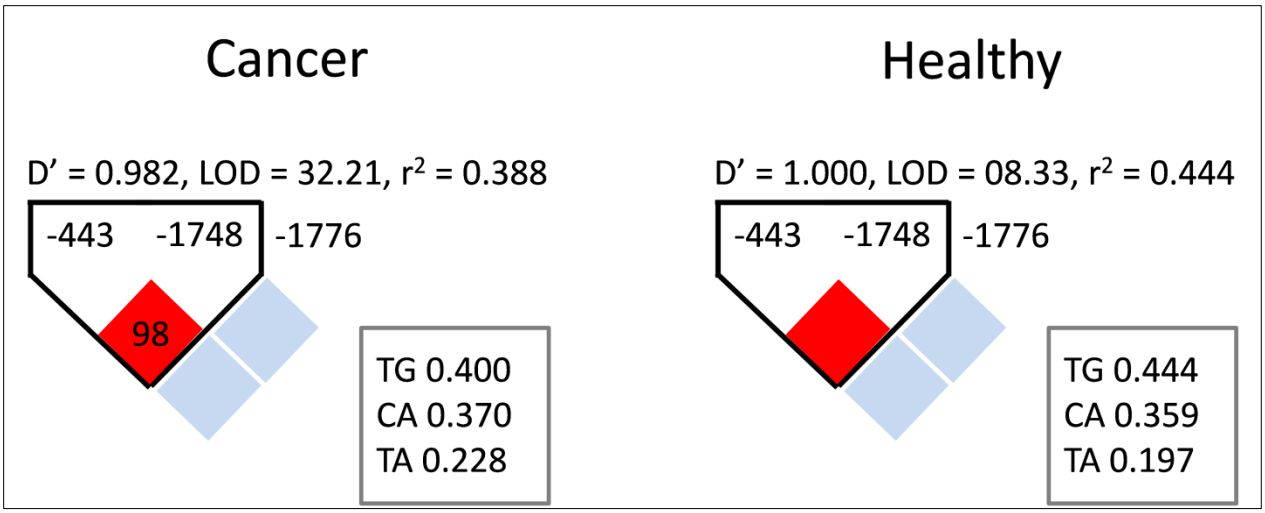



**Figure 3.6 Promoter haplotype.** Haplotype associations were tested for the 4 polymorphic sites under study. The SNP in position −66 was not in Hardy-Weinberg equilibrium and was excluded. In both breast cancer patients and controls there was an association between SNPs −443 and −1748, while no associations were found for SNP −1776. D', normalized linkage disequilibrium coefficient between pairs of loci; LOD, log of the odds score (measure for probability of a linkage relationship among selected loci); r2, squared correlation coefficient between 2 SNPs. SNPs, single nucleotide polymorphisms.

### *3.4. Discussion*

Certain cancer-associated mutations may be individually transforming, such as the chromosome translocation that generates the chimeric kinase BCR-ABL in CML or the loss-of-function mutations of RB that cause retinoblastoma. Other mutations or polymorphisms have the nature of quantitative trait loci that collectively affect the risk for transformation or progression, with each individual site contributing only moderately. It is safe to assume that many of the cancer-associated genetic changes in the latter category have not been identified.

The spp1 gene, which encodes osteopontin is located on chromosome 4q22.1. The very diverse roles of the gene product in physiology and pathophysiology are regulated on the post-transcriptional level (glycosylation, phosphorylation, sulfation, calcium binding, heparin binding, proteolysis, transglutamination), in RNA processing (three alternative splice variants, alternative

translation from a non-canonical start site), and genetically (polymorphisms in coding and non-coding regions). Since 2004 (Giacopelli et al., 2004; Hummelshoj et al., 2006), there has been increasing interest in the biological roles for spp1 promoter polymorphisms in various pathologies. Here we report that promoter polymorphisms are also relevant for breast cancer.

Abundant production of osteopontin is correlated with aggressiveness (higher stage, grade, and early progression) in multiple forms of cancer (Weber et al., 2010). Known mechanisms for osteopontin induction in cancer include the activation of gene expression due to elevated signal transduction and the alternative splicing of the spp1 transcript. It was possible that high expression polymorphisms in the spp1 promoter might also contribute to an elevated risk for tumor aggressiveness. Here we have tested this hypothesis. We found the polymorphic site in position -443 of the promoter to be associated with tumor grade, such that the allele T was more common in high grade tumors. It was also more common in high expressors of osteopontin compared to low expressors. Further, this allele occurred at higher frequency in cancers that lack ER over cancers that express this receptor and in cancers that lack PR over those that express PR. We find T in position -443 is associated with higher aggressiveness of the cancers, and consistently hormone receptor negative cancers tend to grow faster and have a worse prognosis than breast tumors that express ER or PR.

To test our results against the existing knowledge basis, we compared this study to the distribution of polymorphic site frequencies according to public databases (Table 2). The polymorphism in position -443 has been associated with various disease phenotypes (Table 3). A DNA sequence similar to a c-MYB core binding motif, CAGTT, immediately precedes the −443 polymorphic promoter position CAAGTT[C/T]. However, the canonical c-MYB site is 5'-[T/C]AAC[G/T]G-3' (Deng et al., 1996), and transcription via c-MYB from the non-canonical

site in the spp1 promoter may be context-dependent. While c-MYB causes higher transcription from the C allele, there is evidence that under some circumstances the T allele may be associated with higher levels of expression. In melanoma and gastric cancer, the -443 allele C may have elevated transcription over allele T or heterozygous C/T, causing an increased risk for tumor progression and reduced survival rates (Schultz et al., 2009; Zhao et al., 2012). In hepatitis c, the T/T genotype has been associated with an increased anti-viral response to hepatitis c (which requires high levels of osteopontin (Ashkar et al., 2000)), however, the T allele may be more common in patients with high hepatitis activity (reflective of a compromised antiviral response due to low levels of osteopontin secretion) (Mochida et al., 2004; Shaker et al., 2012). In diseases with autoimmune components, the published results likewise point to a complex role for the SNP in position -443. Nephropathy in diabetes is more common in carriers of the T allele (Cheema et al., 2012), which may reflect increased inflammation due to high osteopontin expression. Conversely, thrombocytopenia and hemolytic anemia in lupus have an autoantibody-mediated pathogenesis, which is supported by osteopontin, more strongly in carriers of the C allele (Trivedi et al., 2011). The polymorphic site -443 is associated with osteoarthritis risk and severity. Thrombin-cleaved osteopontin levels in the synovial fluid are lower in subjects with the -443T/T genotype, resulting in milder disease (Jiang et al., 2013). In the present study the T allele is represented more frequently than the C allele at high tumor grade and in tumors with high osteopontin RNA levels (of note, for a subset of samples this result was confirmed by reanalysis in an external core facility to exclude the possibility of an erroneous data set). This implies an important role for c-MYB-independent osteopontin expression in breast cancer.

**Table 3.2 Frequencies of the polymorphisms.** Reported polymorphism frequencies from multiple sources are compared to the distributions obtained in the present study. In ABI, Asians are the average of Japanese and Chinese. For the NCBI SNP, the ratios reflect all studies combined (weighted averages). The present study includes the average of all samples, including cancers, surrounding tissue and healthy controls. SNP, single nucleotide polymorphism; MAF, minor allele frequency; Cauc, Caucasian.

| position | rs number | alleles | HapMap ratio | ABI ratio | NCBI SNP ratio | MAF | this study ratio |
|---|---|---|---|---|---|---|---|
| -1776 | rs29001511 | C/T | 0.017/0.983 | 0.03/0.97 (white)<br>0.00/1.00 (black) | 0.017/0.983 | C=0.0059/13 | 0.007/0.993 |
| -1748 | rs2728127 | A/G | 0.5/0.5 | 0.66/0.34 (white)<br>0.53/0.47 (black) | 0.624/0.376 | G=0.3679/805 | 0.626/0.374 |
| -443 | rs11730582 | C/T | 0.26/0.74 | 0.44/0.56 (white)<br>0.14/0.86 (black)<br>0.305/0.695 (asian) | 0.300/0.700 | C=0.3419/748 | 0.404/0.596 |
| -66 | rs28357094 | G/T | 0.5/0.5 | 0.26/0.74 (white)<br>0.09/0.91 (black) | 0.170/0.830 | G=0.1175/257 | 0.036/0.964 |

| position | rs number | alleles | HapMap ratio | ABI ratio | NCBI SNP ratio | MAF | this study ratio |
|---|---|---|---|---|---|---|---|
| 305 | rs11544546 | A/T | -- | -- | -- | -- | 0.98/0.02 |
| 367 | rs11544549 | C/A | -- | -- | -- | -- | 1.00/0.00 |
| 794 | rs7435825 | G/A | 1.00/0.00 (white)<br>0.81/0.19 (black)<br>1.00/0.00 (asian) | 1.00/0.00 (white)<br>0.88/0.14 (black) | 1.00/0.00 | A=0.043/94 | 1.00/0.00 |
| 1025 | rs4660 | G/A | 1.00/0.00 (white)<br>0.90/0.10 (black)<br>1.00/0.00 (asian) | 1.00/0.00 (white)<br>0.88/0.12 (black) | 0.951/0.049 | A=0.017/38 | 1.00/0.00 |

**Table 3.3 Functions of the polymorphic sites in the spp1 promoter.**

| Position | Transcription Factor | Disease | References |
|---|---|---|---|
| -66 | SP1/SP3 | Duchenne muscular dystrophy | Pegoraro 2011 |
| | | osteoarthritis | Jiang 2013 |
| | | atherosclerosis predisposition | de las Fuentes 2008 |
| | | type 1 diabetes | Giacopelli 2004; Hummelshoj 2006; Marciano 2009 |
| -145/-146 | | nephrolithiasis | Gao 2007 |
| -156 | RUNX2 | glioma | Giacopelli 2004 |
| | | pseudoxanthoma elasticum | Hendig 2007 |
| | | systemic lupus erythematosus, | D'Alfonso 2005; Chen 2010 |
| | | systemic sclerosis | Barizzone 2011 |
| | | diastolic dysfunction in hypertension | Nakayama 2011 |
| -443 | MYB | hepatitis c | Mochida 2004; Shaker 2012 |
| | | melanoma | Schultz 2009 |
| | | gastric cancer | Zhao 2012 |
| | | diabetic nephropathy | Cheema 2012 |
| | | thrombocytopenia, anemia in SLE | Trivedi 2011 |
| | | myocardial infarction | Schmidt-Peterson 2009 |
| | | osteoarthritis | Jiang 2013 |
| -593 | | nephrolithiasis | Gögebakan 2010 |
| -1748 | | pseudoxanthoma elasticum | Hendig 2007 |

The SNP frequency in the osteopontin promoter (table 3.2) is roughly consistent with the estimated variability in DNA sequence among humans of 0.3%. Remarkably, the coding region polymorphisms reported in the NCBI SNP database is disproportionately higher. This is consistent with the low evolutionary preservation of the osteopontin protein sequence and with the low structural constraints of the molecule. It may reflect an unstable chromosome locus. However, few of the deposited SNPs are backed by larger population studies and those located in critical functional sites (such as mutations of the RGD motif) may be exceedingly rare. In this study, we assessed the only non-synonymous (i.e. amino acid-changing) polymorphic sites, for which probes were available. The study population was entirely homozygous for three of the four. Further investigation is required to assess the potential roles of coding region polymorphisms within the spp1 gene in breast cancer. Fifty six of our specimens were assessed

with 2-6-fold coverage. For most of them, the reproducibility was 100%. Few samples with lower quality DNA had reproducibility in 4 of 6 repeats.

# Chapter 4

# Advanced Stage Pancreatic Cancer Progression: A Role for Alternatively Spliced Tissue Factor

# 4. Advanced Stage Pancreatic Cancer Progression: A Role for Alternatively Spliced Tissue Factor

## *4.1. Introduction*

The genetic programs of tumor progression are activated downstream of deregulated growth and survival signals in cancers, but not in benign tumors (Zhang et al., 2003). They direct tissue destruction and dissemination. There is an association between the programs of tumor progression and the hemostatic system (Rickles et al., 2001). Both, coagulopathy and neoangiogenesis are important features of the vascular system associated with malignancy (Falanga et al., 2013; Falanga et al., 2003; Wojtukiewicz et al., 2001). Coagulation disorders ranging from thrombosis to hemorrhage lead to a poor prognosis in cancer patients, and hemostatic system inhibitors have long been prescribed to control tumor progression (Wojtukiewicz et al., 2007). Beyond coagulopathies, the components of the hemostatic system also directly affect the course of the disease via tumor cell proliferation, survival, angiogenesis and metastasis. For instance, in the absence of fibrinogen, lung metastases in mice with melanoma are significantly reduced (Palumbo et al., 2000). Thrombin and its receptor (PAR-1) increase the invasive phenotype in breast cancer (Lal et al., 2013). Tissue Factor (TF) and the complex TF/factor VIIa/factor Xa also play important roles in tumor progression in breast cancer, colorectal cancer, and others (Lal et al., 2013; Palumbo, 2008).

As is known for metastasis genes in general (Weber, 2008; Weber and Ashkar, 2000b), the primary transcript of Tissue Factor, an essential trigger of coagulation, undergoes alternative splicing yielding a secreted variant, termed asTF (alternatively spliced Tissue Factor). Lacking exon 5, asTF is devoid of the transmembrane domain, and is hence soluble and secreted from cells (Bogdanov et al., 2003). Whereas TF expression positively correlates with advanced tumor

stages and thrombosis, asTF does not have a physiologic function in hemostasis, but its expression levels are higher in patients with more aggressive pancreatic ductal adenocarcinoma. asTF expression is associated with increased tumor cell proliferation, metastases and angiogenesis in this cancer (Hobbs et al., 2007; Signaevsky et al., 2008; Unruh et al., 2014), however, the underlying mechanism of tumor progression at the advanced stages, where it is highly expressed, remains unknown.

Carbonic anhydrases catalyze the reversible hydration of carbon dioxide, and thus contribute to pH maintenance. Carbonic anhydrase IX (CAIX) is a transmembrane protein that may be over-expressed in hypoxic cancer cells as a result of increased glycolysis and acidic pH. While increasing survival by preventing an intracellular drop in pH, CAIX in turn makes the extracellular space more acidic and through this process may also affect cancer cell motility. At advanced cancer stages, tumor promoters such as asTF may act through hypoxia-inducible genes, like CAIX.

To understand the mechanism of tumor spread by asTF in advanced stage of pancreatic cancer, asTF-over-expressing pancreatic ductal adenocarcinoma cell line Pt45P1/asTF+ and the parent cell line Pt45P1 were tested for growth and mobility under normoxic conditions (5% $CO_2$, ambient $O_2$, 1000 mg/ml glucose) that model early stage tumors, and in a hypoxic environment that is consistent with advanced-stage cancers (20% $CO_2$, 1% $O_2$, no glucose, 5 mM lactate).

Objective: To analyze the role of alternatively spliced Tissue Factor in tumorigenesis and tumor progression as we mimic the *in vitro* environment for tumor progression from the homeostatic environment of early stage tumors to the hypoxic, acidic, and low-glucose environment of late stage tumors.

Significance: Normal physiologic cell culture conditions aim to impart higher growth capacity to cancer cells, but it is equally important to study how hypoxic culture conditions impart aggressiveness to asTF expressing cells.

### *4.2. Methods*

#### 4.2.1. Cell Culture

The human pancreatic ductal adenocarcinoma cell line Pt45P1 and the asTF-over-expressing transfectant Pt45/asTF+ were cultured in high glucose (1000 mg/l) DMEM supplemented with 10% FBS (Fisher Scientific), HEPES (Gibco), sodium pyruvate (Sigma) and the selecting antibiotic (Zeocin) as appropriate. Early stage cell culture conditions (normoxic, physiologic pH, high glucose) were characterized by 5% $CO_2$, ambient $O_2$, 1000 mg/l glucose and 0 mM lactate, whereas advanced stage conditions (hypoxic, low glucose/high lactate) comprised 20% $CO_2$, 1% $O_2$, 0 mg/l glucose and 5 mM lactate in BME medium (Gibco).

#### 4.2.2. Protein Expression

Cell lysates were collected 48 h after plating the cells at 0.5 x $10^5$/well of a 24-well plate under early or advanced stage conditions. For generating chemical hypoxia as a positive control, HeLa cells were treated with 100 μM $CoCl_2$ for 48 h before lysing. The Pierce BCA assay was performed to determine protein concentration, and 15 μg of protein was loaded per lane of 8% SDS PAGE. The separated proteins were then transferred to a PVDF membrane, blocked with 5% nonfat milk, probed with the rabbit monoclonal asTF RabMab1 (Srinivasan et al., 2011), rabbit mAb vimentin (Cell Signaling), rabbit β-actin(Cell Signaling), rabbit pAb HIF-1α (Bethyl Laboratories), rabbit pAb HIF-2α (GeneTex) or mouse mAb CAIX (GeneTex), followed by

probing with its corresponding HRP-conjugated secondary antibody (Bio-Rad) and developed using ECL and H2O2.

### 4.2.3. Cell Proliferation Assay

Triplicates of Pt45P1 and Pt45P1/asTF+ cells were plated under early stage or advanced stage conditions in 96-well plates at a density of 2,000 cells/well. The cell abundance was measured on days 0, 1, 2, 3 and 4 using Cell Proliferation Reagent WST-1 (Roche), according to the manufacturer's protocol. WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) is taken up by life cells and is cleaved by enzymes of the intermediary metabolism to formazan, which is retained intracellularly. This generates an absorbance at 420-480 nm and can be directly correlated to the viable cells in the system.

### 4.2.4. Cell Cycle Analysis

Pt45P1 and Pt45P1/asTF+ stable transfectants were plated in 24-well plates either for adhesion (directly on the plastic surface of the plate) or under deadherent conditions (on a layer of 0.015 $\mu g/mm^2$ polyHEMA (Kuroda et al., 2013)), at a minimum density of 100,000 cells/well. 72 h after plating, the cells were harvested and stained with propidium iodide (Sigma) (Adler et al., 1998), and were analyzed for cell cycle stage with a FACS Calibur flow cytometer (BD Biosciences).

### 4.2.5. Gap Closure (Wound Healing) Assay.

Pt45P1 and Pt45P1/asTF+ cells were plated in 12-well plates at a density of 0.1 x 106 cells/well in early stage or advanced stage environments. After 1-1.5 days, the cells had adhered and reached confluence, the plates were scratched at the center of each well using a P200 pipet tip. The assay was performed in the presence or absence of 2 μM thymidine (a G1/S blocker of cell

cycle progression). Photographs were taken at 0, 18, 24, 48, 72 and 96 h. The wells were quantitatively analyzed for gap closure activity by determining the area left unoccupied using the software ImageJ.

#### 4.2.6. Pharmacologic Inhibition of Carbonic Anhydrase IX

To evaluate the effect of CAIX inhibition on asTF mediated tumor progression, functional assays were performed in the presence of the carbonic anhydrase IX and XII inhibitor U-104 (EMD Millipore) at a concentration of 75 μM or vehicle control (0.1% DMSO). The effect was tested under early and advanced stage environments using both Pt45P1 and Pt45P1/asTF+ cells.

### *4.3. Results*

#### 4.3.1. CAIX is a downstream target of asTF

As asTF expression is significantly upregulated in patients with advanced stage pancreatic cancer (Unruh et al., 2014), it is important to identify downstream mechanisms, through which it may promote tumor progression. Whereas conventional cell culture conditions aim to impart high growth capacity to cancer cells, they do not suitably model the microenvironment of advanced cancers. We therefore analyzed Pt45P1/asTF+ and Pt45P1 lysates for upregulated expression of specific proteins under normoxic conditions that model early stage tumors and in the hypoxic environment of advanced-stage cancers. Although both Pt45P1/asTF+ cells had similar expression levels of HIF-1α, we found hypoxia-induced expression of CAIX (downstream target of HIF-1α) selectively by Pt45P1/asTF+ cells. By contrast, Pt45P1 cells had only a weak expression of CAIX, even when cultured in an advanced stage environment (Fig. 4.1). CAIX is an enzyme inducible by the lack of oxygen that plays important roles in maintaining intracellular pH and increasing cancer cell invasiveness. HIF-2α was selectively expressed by Pt45P1 cells

with a basal level of asTF expression. Over-expression of asTF was accompanied by negligible HIF-2α expression.

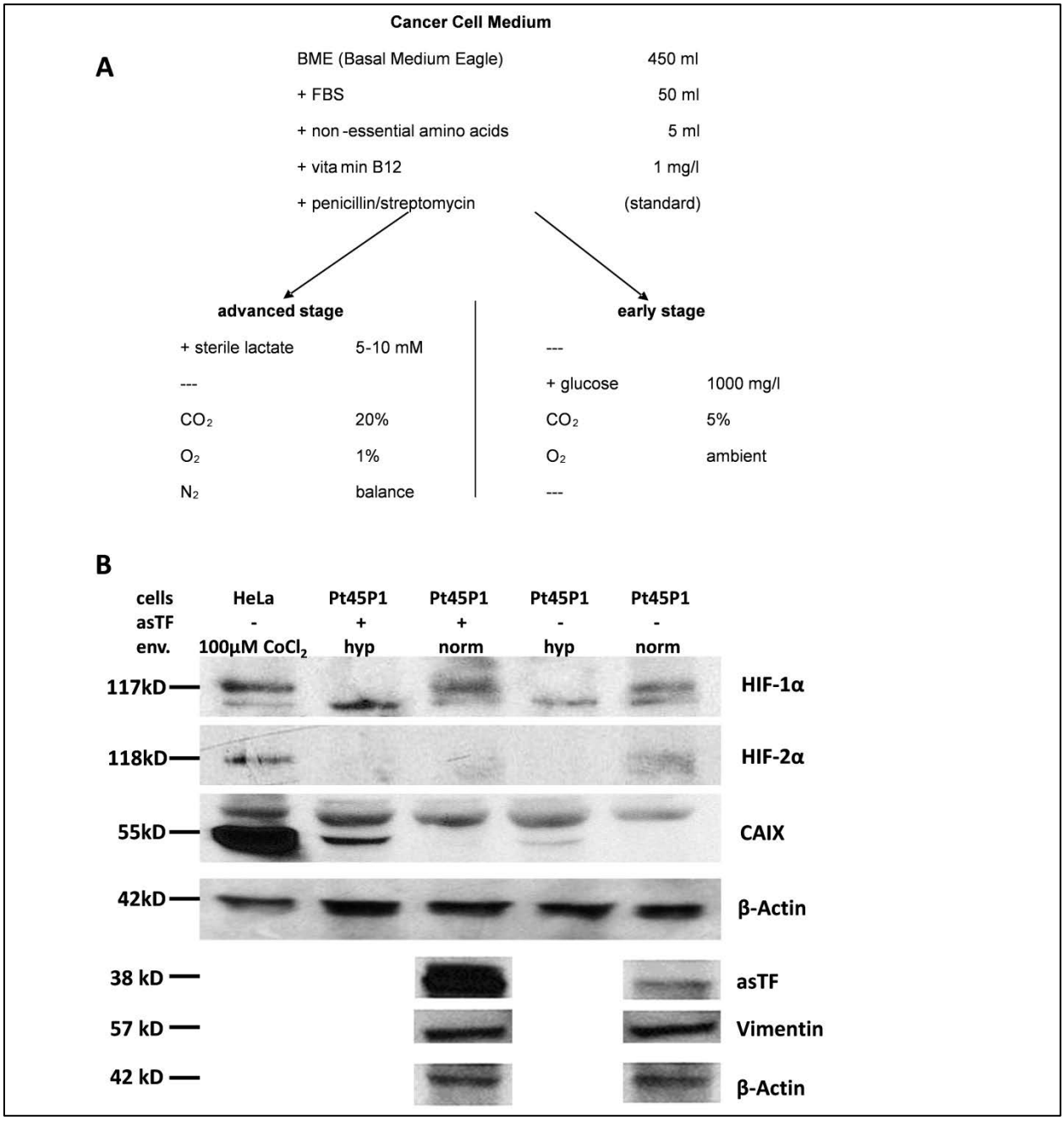



**Figure 4.1 Up-regulation of CAIX by asTF over-expression.** A) cell culture conditions for modeling early stage or late stage pancreatic cancer progression. B) Expression of the hypoxia-associated proteins HIF-1α (117 kD), HIF-2α (118 kD), and CAIX (55 kD) in early (norm) or late stage (hyp) environments. β-Actin served as a loading control. HeLa cells treated with 100 µM CoCl2 were used as a positive control for hypoxia-induced gene expression. env. represents the environment, under which the cells were kept for 48 hours before lysing, with hyp indicating hypoxic/low glucose and norm indicating normoxic/high glucose. The bottom panel shows the expression in Pt45P1 cells of the transfected gene asTF, vimentin (Sipos et al., 2003), and the loading control β-Actin.

### 4.3.2. asTF induces cell cycle progression, CAIX plays a contributing role

To test the effect of asTF expression on cell division, Pt45P1 and Pt45/asTF+ cells were assessed for cell proliferation using a colorimetric assay. Over a period of four days Pt45/asTF+ cells showed a significantly higher rate of proliferation under both early and advanced stage conditions than Pt45P1 cells (figure 4.2). U-104 did not affect cell division of Pt45P1 cells under either early or advanced stage environments.

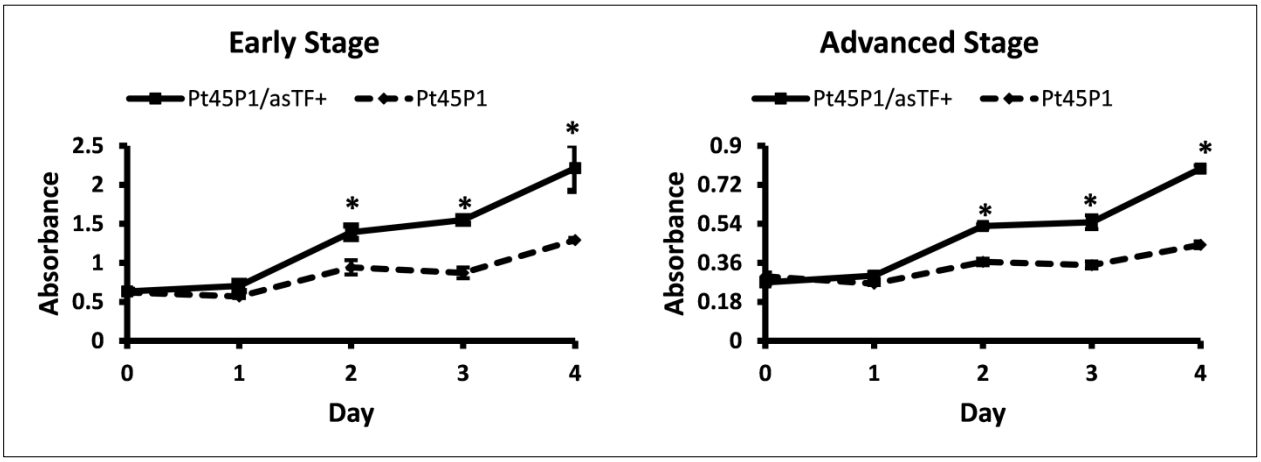



**Figure 4.2 Cell Proliferation in normoxia and hypoxia.** Pt45P1 and Pt45P1/asTF+ cells were plated in triplicates in 96-well plates in A) the early stage environment (5% CO2, ambient O2, 1000 mg/l glucose, 0 mM lactate) or B) the advanced stage environment (20% CO2, 1% O2, 0 mg/l glucose, 5 mM lactate). *indicates significance $p < 0.05$.

To further corroborate the proliferation results, Pt45P1 and Pt45/asTF+ cells, plated under early and advanced stage conditions, were analyzed for cell cycle stage via propidium iodide staining and flow cytometry. While Pt45P1 cells had a significantly higher percentage of cells in the non-dividing $G_0/G_1$ phase, asTF-over-expressing cells were more prominently found in the $G_2/M$ phase of the cell cycle under both early (figure 4.3a) and advanced (figure 4.3b) stages. Tumor progression, under conditions of deadhesion, requires cell survival and cell growth. Therefore, to determine whether asTF expression contributes to cell growth under deadherent conditions, both Pt45P1 and Pt45/asTF+ cells were plated on a layer of polyHEMA to prevent the cells from

attaching to the plastic surface of the cell culture dishes. Cells over-expressing asTF had a significantly higher percentage of cells dividing, even under these conditions, in both early stage (figure 4.3c) and advanced stage environments (figure 3d). Thus, asTF expression promotes cell growth under adhesive conditions that model cell attachment to the basement membrane, as well as in deadhesive states that represent cancer cells in circulation.

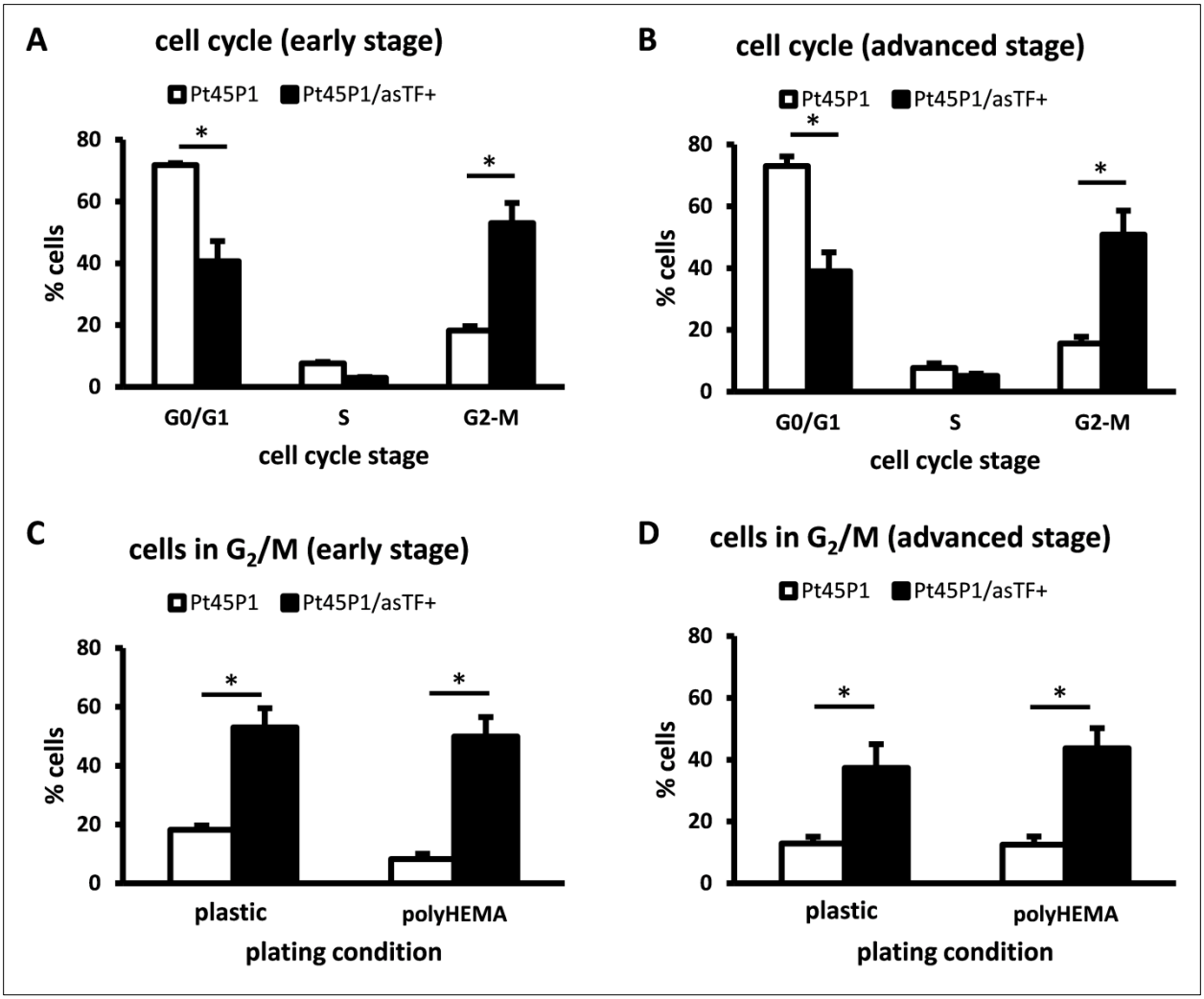



**Figure 4.3 Effect of asTF on cell cycle phases.** Pt45P1 and Pt45P1/asTF+ cells were plated in triplicates in early stage (A) or advanced stage (B) environments and were analyzed for cell cycle stage via flow cytometry after propidium iodide staining. The percentage of cells in G2/M was assessed after plating in conventional cell culture dishes (plastic) or on poly-HEMA (polyHEMA) to prevent cell adhesion. The comparison was done under early stage (C) or advanced stage (D) conditions. * indicates significance at $p < 0.05$.

U-104, a benzene-sulphonamide, is a pharmacologic inhibitor of carbonic anhydrase IX and XII (Lock et al., 2013; Lou et al., 2011). The cell proliferation rate of asTF-over-expressors under advanced stage conditions was decreased in the presence of U-104 (figure 4.4). When CAIX was inhibited by U-104 in adherent cells under advanced stage conditions, the percentage of Pt45P1/asTF+ cells in the $G_0/G_1$ phase significantly increased by 44% as compared to Pt45P1/asTF+ cells without drug treatment (figure 4.5). This inhibition via U-104 also reduced the percentage of cells in the $G_2$/M phase of the cell cycle in the advanced stage environment. The drug did not affect the cell cycle distribution in Pt45P1/asTF+ cells in early stage or of Pt45P1 cells in early or advanced stages.

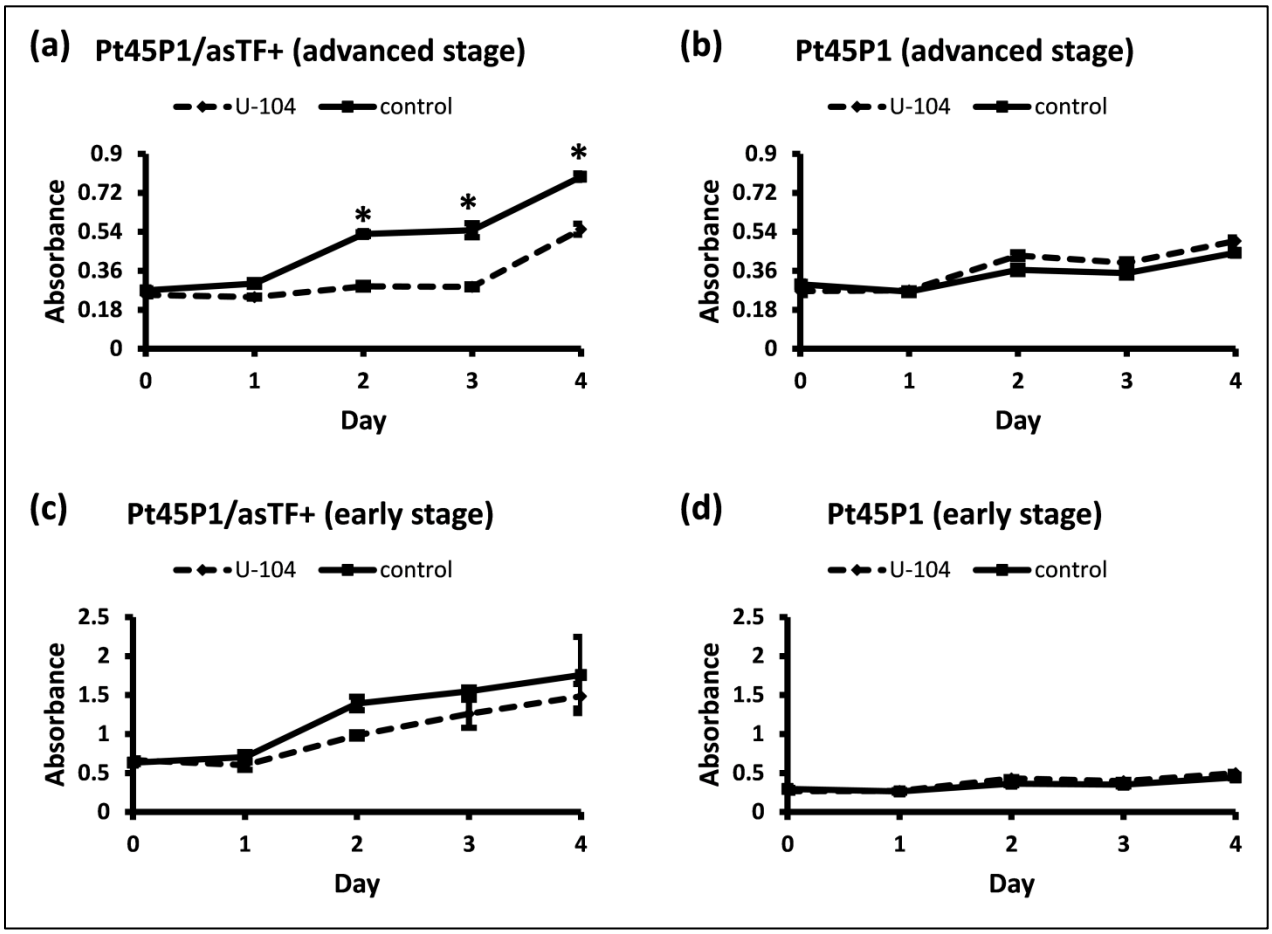



**Figure 4.4 Effect of CAIX inhibition on cell growth.** Effects of the CAIX inhibitor U-104 on cell proliferation by Pt45P1/asTF+ cells (A,C) or Pt45P1 cells (B,D) under advanced stage (A,B) or early stage conditions (C,D). At the indicated times, WST-1 uptake was measured by colorimetry. The error bars are sem. * indicates significance at $p < 0.05$.

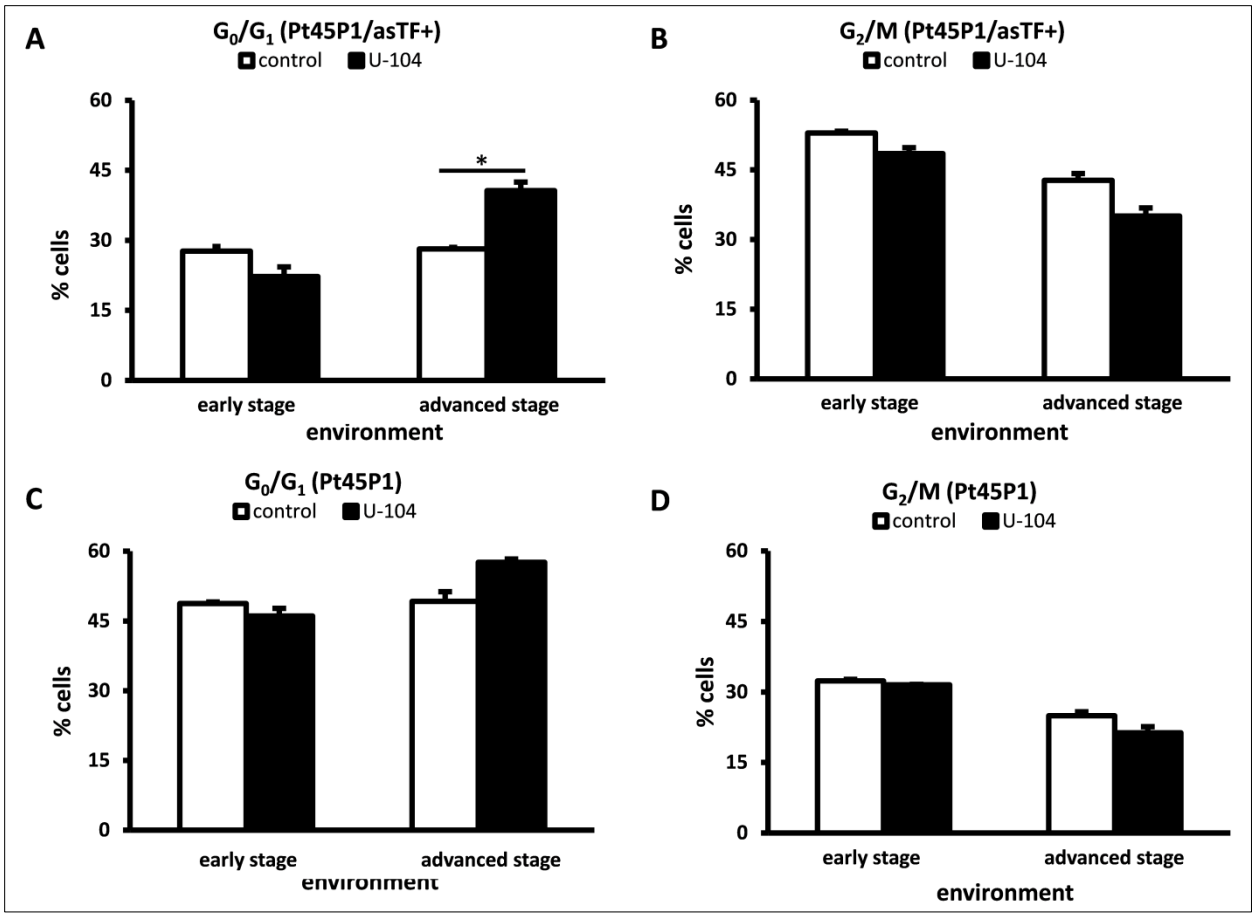



**Figure 4.5 CAIX inhibition decreases cell cycle progression.** The effect of U-104 on cell cycle progression was assessed by flow cytometry after propidium iodide staining. A,C) Percentage of cells in the G0/G1 phase of the cell cycle for Pt45P1 cells (A) and Pt45P1/asTF+ cells (C). B,D) Percentage of cells in the G2/M phase of the cell cycle for Pt45P1 cells (B) and Pt45P1/asTF+ cells (D). The error bars are sem. * indicates significance at $p < 0.05$.

### 4.3.3. asTF expression imparts higher mobility to Pt45 cells, in hypoxia CAIX is a mediator

A gap closure (wound healing) assay was performed to assess the ability of asTF-over-expressing Pt45P1 cells to close the gap created by disrupting a monolayer through scratching the center of a well. asTF over-expression facilitated cell motility to close the gap and thus a faster wound healing ability (figure 4.6). The experiment was also performed in the presence of 2 μM thymidine, which blocks cell cycle progression at $G_1$/S phase (figure 4.7), to ensure that the gap closure is due to cell motility and not cell division (Rosner et al., 2013). To evaluate the

importance of CAIX, which acts downstream of asTF in pancreatic cancer, cell mobility assays were performed in the absence or presence of U-104. In the presence of U-104, the ability of Pt45P1/asTF+ cells to close the gap was significantly reduced as compared to untreated or DMSO treated cells under advanced stage sonditions (figure 4.8). U-104 neither affected the mobility of Pt45P1 cells (under early and advanced stage environments), nor Pt45P1/asTF+ cells under early stage conditions. Thus, the hypoxia-specific expression of CAIX by asTF-over-expressing cells imparts them with a more aggressive phenotype under the hypoxic, acidic conditions representing the advanced tumor grades seen in patients.

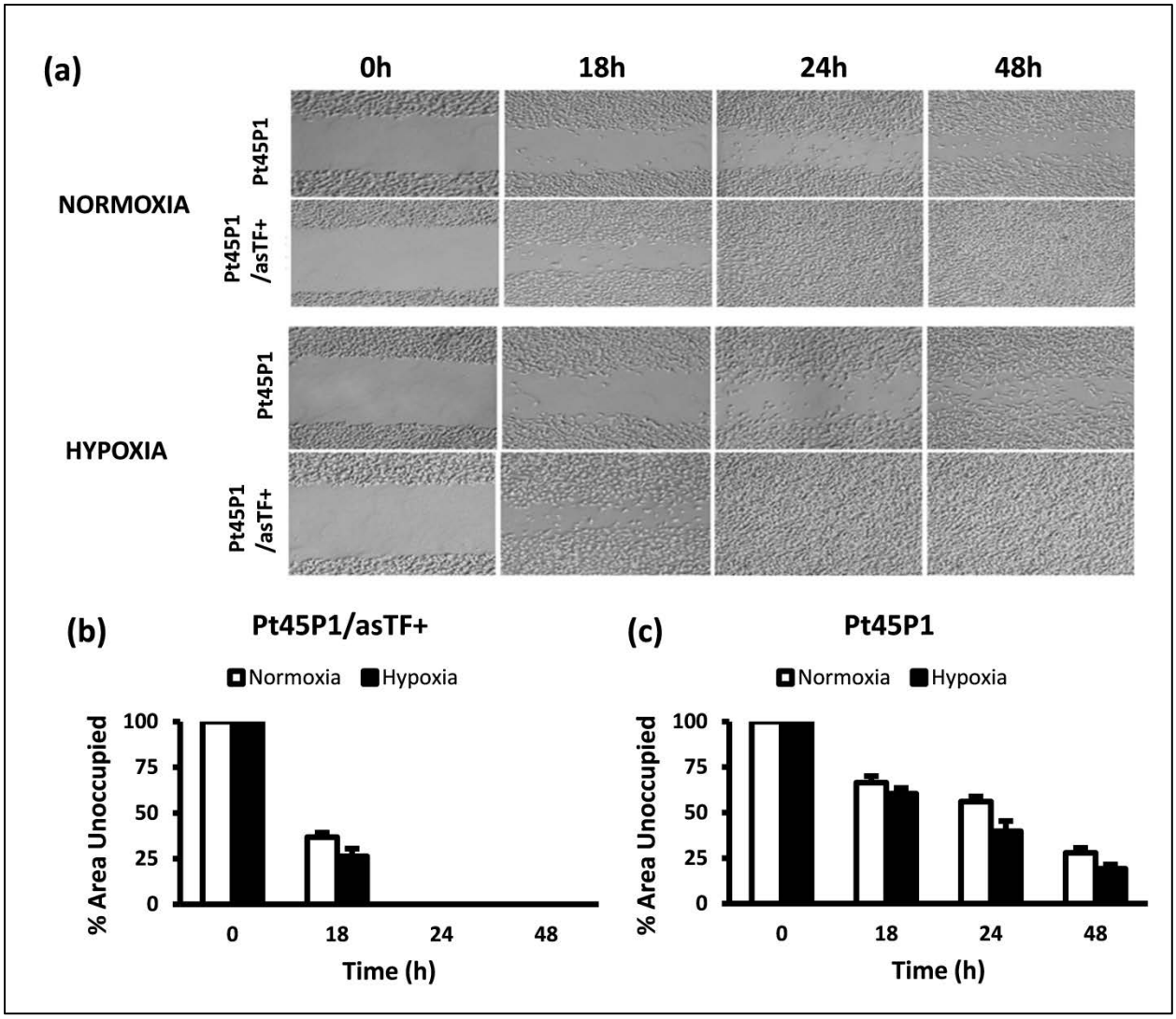



**Figure 4.6 Acceleration of gap closure by asTF.** (a) A comparison of gap closure ability of asTF over-expressors versus Pt45P1 cells under early and advanced stages. (b, c) Quantification of Cell motility assay. This graph represents the area unoccupied by Pt45P1/asTF+ and Pt45P1 cells under both early and advanced stages at various time intervals (0 h, 18 h, 24 h, 48 h).

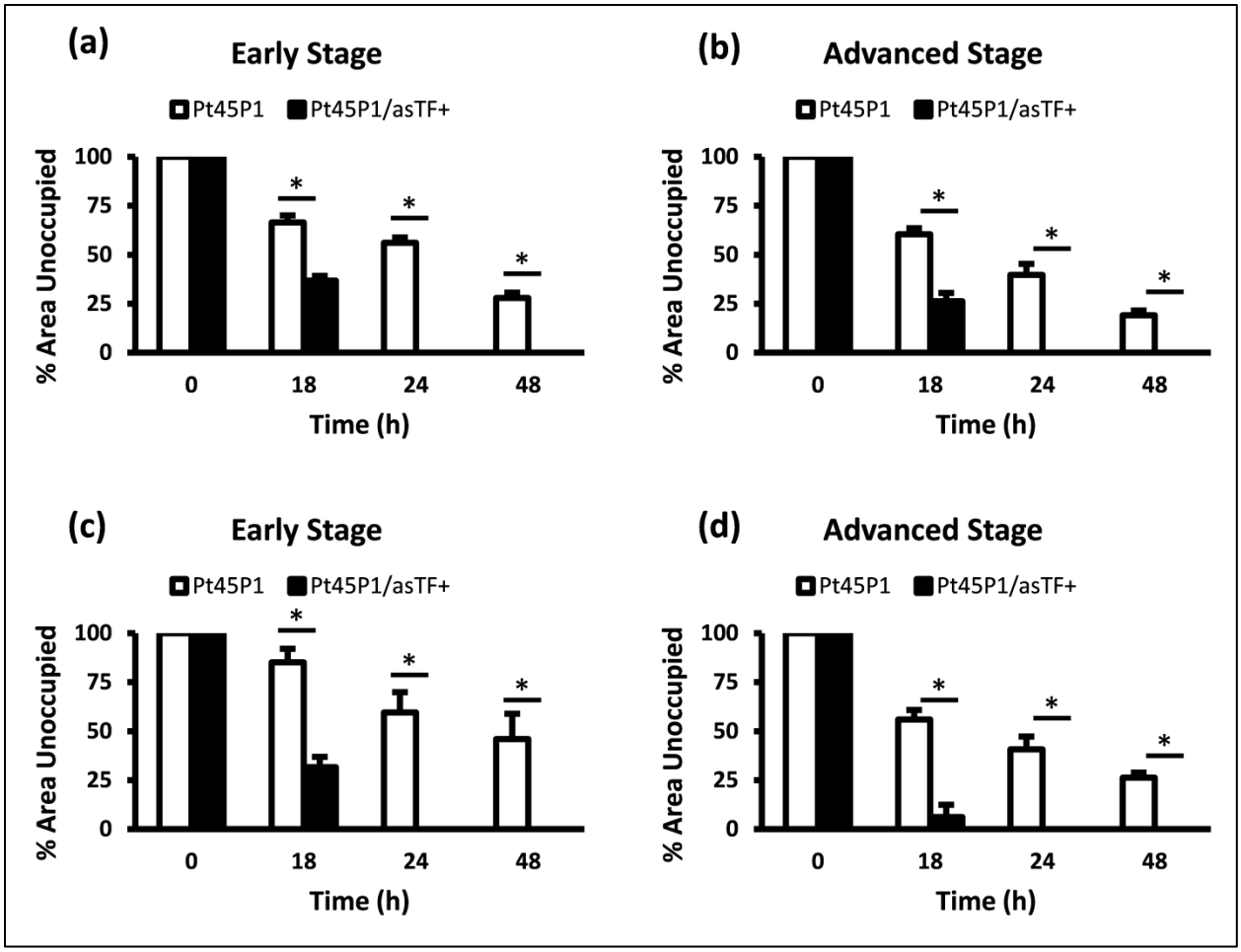



**Figure 4.7 Effect of $G_1$/S Blocker.** (a, b) This assay was performed to compare the gap closure ability of asTF over-expressors and Pt45P1 cells in the presence of thymidine (2 μM), $G_1$/S blocker. (b, c) Quantification of cell motility assay in the absence of thymidine. The graph represents the area unoccupied by Pt45P1/asTF+ and Pt45P1 cells under both normoxia and hypoxia at various time intervals (0 h, 18 h, 24 h, 48 h). *indicates significance at $p<0.05$.

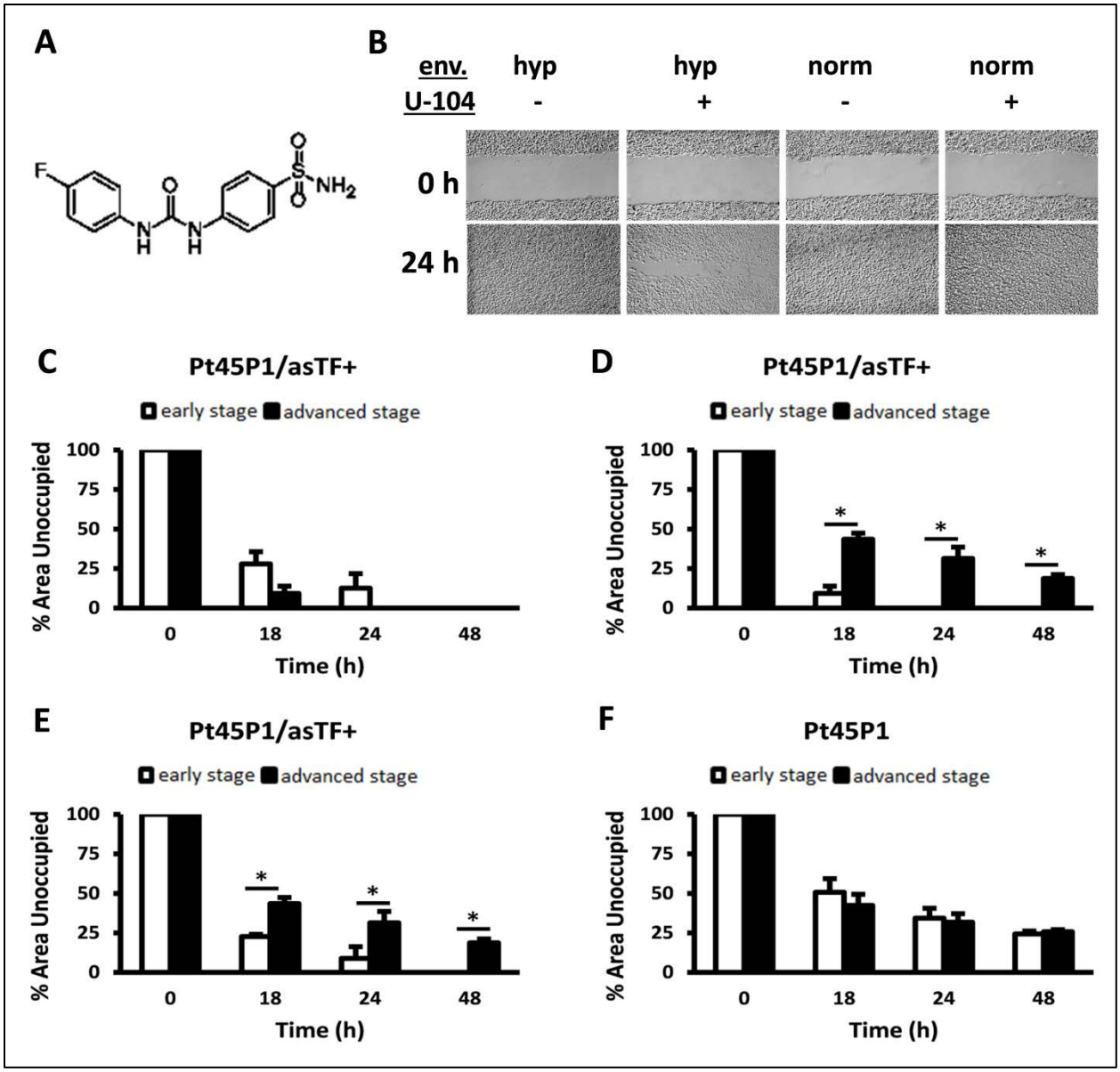



**Figure 4.8 Effect of CAIX inhibition on gap closure.** (A) structure of the CAIX inhibitor U-104, 1-(4-fluorophenyl)-3-(4-sulfamoylphenyl)urea. (B) In the presence of CAIX inhibitor (U-104), the gap closure ability of Pt45P1/asTF+ cells was much lower under advanced stage when compared to the same cells in the presence of control (DMSO) or its early stage counterpart. In the presence of U-104 (75 μM), Pt45P1/asTF+ cells were not able to close the gap completely under advanced stage after 24 h as they did in the absence of the drug. (c,d,e,f) The graphs quantify the gap closure ability of Pt45P1 and Pt45P1/asTF+ cells in the presence or absence of U-104. In the presence of DMSO (vehicle only), the gap closure activity of Pt45P1/asTF+ cells remains unaffected under both early and advanced stages (C). Only the mobility of Pt45P1/asTF+ cells is significantly decreased in the presence of U-104, when compared to DMSO-treated cells (D) and is only decreased under advanced stage (E), whereas Pt45P1 cells remain unaffected by U-104 treatment (F). *indicates significance at $p<0.05$.

### *4.4.Discussion*

In the hypoxic tumor environment at advanced pancreatic ductal adenocarcinoma stages, asTF expression is prominent in tumor lesions, surrounding tumor tissues, and lymph nodes. It may correlate positively with tumor stage (Unruh et al., 2014). Here we confirm that asTF supports proliferation and migration, thus enhancing aggressive behavior. Although the in vitro phenotype of tumor growth and motility by asTF over-expression is not very different under advanced stage conditions from that under early stage conditions, these environments are associated with distinct downstream signaling pathways that do (hypoxia) or do not (normoxia) involve CAIX.

CAIX is a downstream mediator of the effects of activated HIF-1α under advanced stage culture conditions. Although HIF-2α expression is seen in Pt45P1 cells, it is not associated with asTF over-expression. This difference in the expression levels of hypoxia-inducible factors due to difference in the expression levels of asTF could be explained by the fact that both HIF-1α and HIF-2α regulate different pathways under hypoxia (Ratcliffe, 2007). It needs to be further elucidated how asTF expression levels are able to modify HIF-2α expression.

Because glycolysis constitutes a common metabolic pathway in cancer cells that leads to the generation and accumulation of high levels of lactic acid, the intracellular pH of these cells drops substantially. CAIX is a membrane-bound enzyme that catalyzes the conversion of water and carbon dioxide, extracellularly, to bicarbonate ions and protons. These bicarbonate ions are then transported inside the cells, bringing up the intracellular pH close to physiologic levels, so that cell survival is assured. Through the same process, CAIX is known to cause an accumulation of protons extracellularly, which may further make the cells motile.

For over six decades, cancer cells have been grown in culture (Gey et al., 1952), and the conditions have conventionally been devised to maximize cell growth. Such systems disregard one of the important pathophysiological properties of advanced tumors: the prevalence of hypoxia (Vaupel and Mayer, 2007). During early stages of transformation, tumor cells acquire gain-of-function mutations in oncogenes or loss-of-function mutations in tumor suppressor genes that cause excessive proliferation and anti-apoptosis. As tumor cells multiply, they outgrow the diffusion limits of oxygen and glucose, thus becoming hypoxic and energy-deprived. Because of increased glycolysis, more lactic acid is generated which makes the lesions acidic. Even though new blood vessels are formed in cancer angiogenesis, they are disorganized and cannot effectively alleviate hypoxia (Carmeliet and Jain, 2000). The cells then undergo cell death or adapt to a glycolytic phenotype. The decrease in pH also leads to a selection of motile cells that breach the basement membrane and cause metastasis, perpetuating a vicious circle (Gatenby et al., 2007). Due to uncontrolled cell proliferation and increased glycolysis by cancer cells, advanced stages of tumor are more hypoxic, acidic and low in glucose as compared to normal physiologic conditions used in cell culture (Mayer and Vaupel, 2013; Osinsky et al., 2009). Such conditions need to be studied in model systems that account for the changes in microenvironment during tumor progression.

Although cell culture is a highly valuable technique in cancer research, it has important limitations for modeling in situ conditions. The technique has been developed in a direction that allows high signal-to-noise ratios, which often exceed physiologic changes. Plastic dishes render cells hypo-active, so that their transcriptional baseline activity is reduced compared to cell growth on extracellular matrix molecules and responses to external stimuli may be exaggerated (Syed et al., 2008). Likely in part because of the baseline-suppressing culture conditions, gene

expression changes by cell lines in response to environmental stimuli have a disproportionately higher magnitude than similar changes in vivo (Weber et al., 2014). While normoxic cell culture conditions have been traditionally designed to maximize cell growth, an aggressive phenotype that may arise in an advanced-stage micro-environment prompted us to study the mechanisms that might promote invasive behavior under hypoxic tumor conditions.

In patients with pancreatic ductal adenocarcinoma, asTF expression in lesions as well as the surrounding tumor environment has been shown to correlate positively with tumor stage (Unruh et al., 2014). Under hypoxic tumor environment at advanced PDAC stages, asTF expression is prominent in tumor lesions, surrounding tumor tissues and lymph nodes. Therefore, it becomes necessary to understand the importance of asTF expression under the harsh, hypoxic and acidic environment of late-stage cancers. Our results demonstrate that although the in vitro phenotype of tumor growth and progression by asTF over-expression under advanced stage conditions is not very different from that under early stage conditions, although cancer cell growth and motility can be reduced via CAIX inhibition. CAIX is a hypoxia-inducible enzyme, specifically expressed by asTF over-expressors and plays an important role in mediating tumor growth and progression under the non-conducive hypoxic environment of late-stage cancers.

Since glycolysis is the common metabolic pathway in cancer cells that leads to generation and accumulation of high levels of lactic acid, the intracellular pH of these cells drops drastically. This acidic environment might aid in making the cells motile under hypoxia, eventually aiding in cancer metastasis.

Lock et al. (Lock et al., 2013) and Lou et al. (Lou et al., 2011) have previously described the use of U-104 (structure shown below) in the pharmacological inhibition of CAIX both in vitro and in

vivo. A benzenesulphonamide, U-104, specifically inhibits CAXII ($IC_{50}$ = 4.5 nM) and CAIX ($IC_{50}$ = 45.1 nM), whereas has only a weak inhibitory effect on other carbonic anhydrases, if at all (CAI: $IC_{50}$ = 5.08 μM and CAII: $IC_{50}$ = 9.64 μM). CAIX inhibition via U-104 has shown to significantly decrease tumor growth in breast cancer. In our in vitro hypoxia model, we did see significant reduction in pancreatic cancer cell growth in the presence of U-104 inhibitor. This inhibition was limited to CAIX over-expressing Pt45P1/asTF+ cells, while Pt45P1 cells had always lesser tumor growth when compared to over-expressors, both in the presence and absence of U-104.

# Chapter 5

# The role of OPN splice variants under tumor Hypoxia

# 5. The role of OPN splice variants under tumor Hypoxia

## *5.1. Introduction*

Transformed cells show excessive proliferation, extension of lifespan and metastatic spread (Weber, 2008). Gain-of-function mutations of oncogenes and loss-of-function mutations of tumor suppressor genes are responsible for the uncontrolled proliferation of transformed cells during cancer initiation. Over-activity of senescence suppressor genes and inactivity of senescence genes underlies extension of life span of transformed cells. Finally, it is the metastasis genes that are responsible for the ability of cancer cells to disseminate. The metastasis gene osteopontin has been associated with the invasiveness of various cancers. Out of the three known splice forms of osteopontin, OPNc has been shown to be a specific marker for tumor progression (Mirza et al., 2008) and also to induce anchorage-independent growth in breast cancer cells (He et al., 2006). While these effects confirm a role of OPN in the acquisition of aggressiveness, contributions of OPN splice forms to later stages of cancer, characterized by low oxygen tension, low pH and limited glucose availability, are unknown.

## *5.2. Methods*

### 5.2.1. Cell Culture

MCF-7 cells transfected with OPNa, OPNc or an empty vector control were grown in αMEM with insulin and 10% fetal bovine serum. MDA-MB-435 cells that endogenously express all three splice forms of OPN were cultured as described previously (Seraj et al., 2000). The normoxic and hypoxic conditions that were used to mimic early and late stages of cancer, respectively, are described in table 5.1.

**Table 5.1 Cell Culture Conditions.** A comparison of early stage, normoxic, intermediate hypoxic and advanced stage, hypoxic cell culture media.

| | Early Stage | Intermediate Stage | Advanced Stage |
|---|---|---|---|
| **Carbon dioxide** | 5% | 10% | 20% |
| **Oxygen** | Ambient | 5% | 1% |
| **Glucose** | 1,000 mg/l | 300 mg/l | 0 mg/l |
| **Lactic acid** | 0 mM | 3 mM | 5 mM |

### 5.2.2. Expression of OPN

For the analysis of secreted OPN in cultured MCF-7 and MDA-MB-435 cells, the serum-free cell culture supernatant was collected from each cell type. 40 µl of supernatant for each cell type was used for OPN expression on a 10% non-reducing SDS-PAGE.

### 5.2.3. Assessment of Cell Proliferation Rate

MCF-7 OPNa, OPNc and empty vector transfectants were plated under the different normoxic and hypoxic conditions. The cell proliferation rate was analyzed by using an automated counter (Cellometer Vision, Nexcelom Bioscience) at four different time points: 0 h, 24 h, 48 h and 72 h.

### 5.2.4. Colony Formation Assays

For all cell types, the ability of soft agar colony formation was analyzed under all the microenvironments representing early and late stages of cancer. 1 x $10^5$ cells were plated per 60 mm dish in triplicates on a top layer of 0.3% agar and a bottom layer of 0.5% agar. 0.4 ml of medium was supplemented every alternate day and the cells were examined for their size of

colonies after 1-2 weeks. The size of the colonies formed was quantified using the software, ImageJ.

### 5.2.5. Cell Cycle Stage Analysis

The MCF-7 transfectants were analyzed for cell death and division under adherent and de-adherent (poly-HEMA) conditions under early and late stages of tumor. 72 hours after plating, the cells were harvested, stained with propidium iodide and analyzed using FACS Calibur. Cells plated directly on plates in the presence of 700 μM hydrogen peroxide, served as positive control for cell death.

### 5.2.6. Effect of Oxidative Stress on OPN splice forms

Upon observation of different phenotypes in the presence of $H_2O_2$ by OPN splice variants during cell cycle stage analysis, MCF-7 OPN transfectants were plated in the presence of varying concentrations of hydrogen peroxide (ranging from 0 to 1000 μM) for 72 hours. these were then harvested, stained with propidium iodide and analyzed by FACS Calibur for percentage of cells undergoing apoptosis.

## *5.3. Results*

### 5.3.1. Colony Formation

There are two components to anchorage-independence: cell growth and cell survival (anti-anoikis). Anchorage-independent cell growth can be assessed by the ability of transformed cells to form colonies in a viscous medium, like colony formation in soft agar, independent of attachment to a solid substratum. Cell survival can be tested by the percent cells undergoing apoptosis in the absence of a solid support.

Our initial experiments testing the effect on soft agar colony formation by different OPN transfectants in MCF-7 cells (non-aggressive breast cancer cell line, not expressing any endogenous osteopontin) under normoxic and hypoxic tumor conditions show that cells expressing OPNc have the ability to form larger clones in soft agar under both normoxic as well as hypoxic tumor conditions over OPNa transfectants and empty vector controls (Figure 5.1).

Figure 5.1 shows that the ability to form bigger colonies decreases as the culture environment progresses from normoxic (ambient $O_2$, 5% $CO_2$, 1000mg/l glucose) through intermediate hypoxic (5% $O_2$, 10% $CO_2$, 300mg/l glucose, 3mM lactic acid) to hypoxic (1% $O_2$, 20% $CO_2$, 5mM lactic acid) conditions. The colonies formed under hypoxic conditions were significantly less in both size and number of cells per colony and although OPNc still formed slightly larger colonies than OPNa and vector, there were no statistically significant differences between the three transfectants under this environment. Because of this, further soft agar colony formation experiments were performed only under normoxic and intermediate hypoxic conditions. For normoxic and intermediate hypoxic environments, we found that OPNc transfected cells retained their ability to form significantly larger colonies over both OPNa transfected cells and empty vector controls.

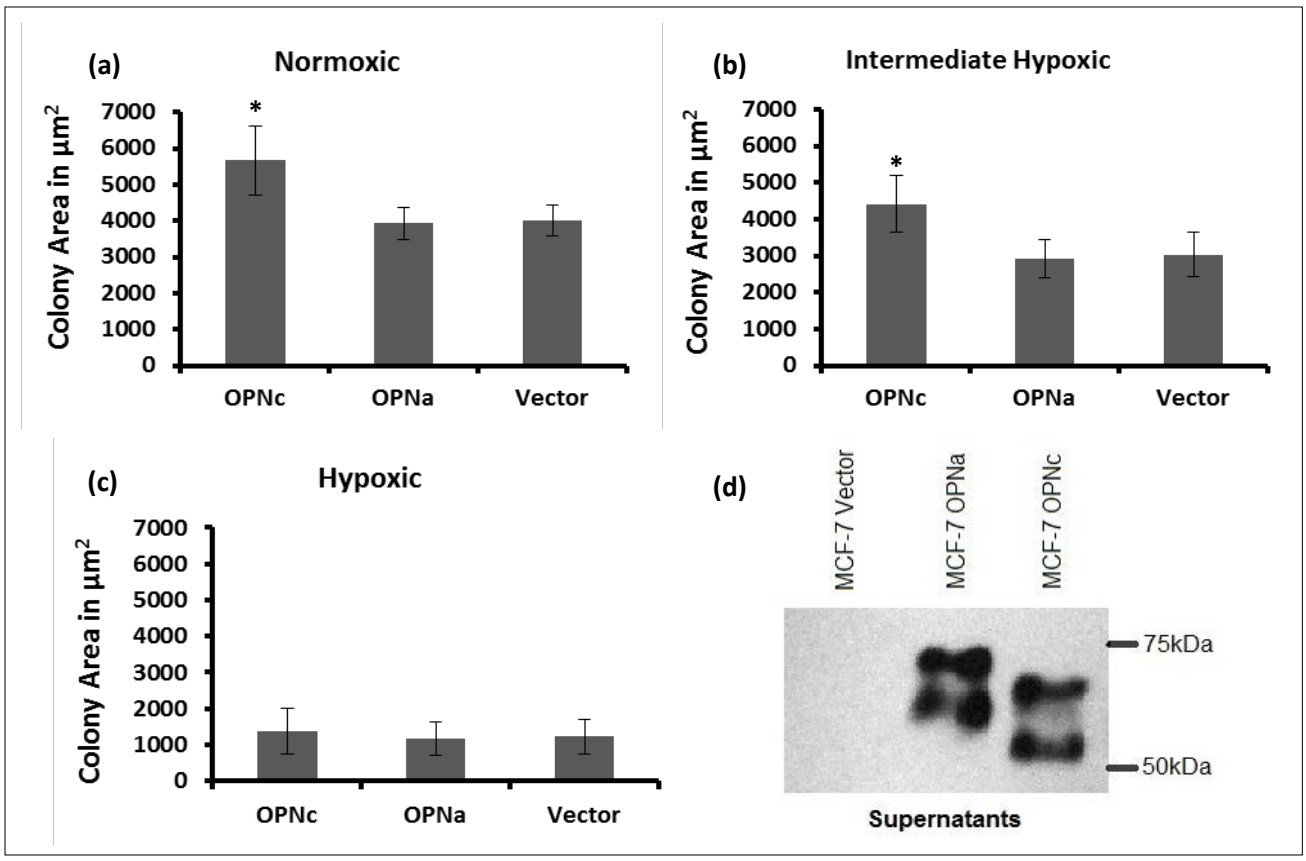



**Figure 5.1 Anchorage-independent growth.** (a) Comparison of soft agar colony formation by MCF-7 transfectants OPNc, OPNa & empty vector under early stage, normoxic environment. MCF-7 cells expressing OPNc formed largest clones. Results are an average of eight separate experiments. Colony area in $\mu m^2$ (mean ±S.E.) (b) The ability to form larger clones was protected under intermediate hypoxic cancer conditions by cells expressing OPNc. Results are an average of six separate experiments. Colony area in $\mu m^2$ (mean ±S.E.) (c) At extreme hypoxic conditions the ability to form colonies was drastically reduced for all three transfectants and no significant differences were seen for colonies formed by any of the three transfectants. Results are an average of two separate experiments. Colony area in $\mu m^2$ (mean ±S.E.) *Significance $p < 0.05$. (d) Western Blot expression of OPN transfectants in MCF-7 cells. The supernatants were collected after starving the cells in serum-free media for 24h. The detection of two bands on Western Blot after transfection has been observed before.

Next, to check whether the presence of endogenously produced OPN is important for anchorage-independent growth in breast cancer cells, MDA-MB-435 cells (aggressive breast cancer cell line, expressing endogenous OPN) were plated for soft agar assay in the absence or presence of OPN-neutralizing (Goat polyclonal IgG OPN antibody, AF1433, R&D systems) or control IgG (AB-108-C, R&D systems) antibody, under both normoxic and intermediate hypoxic conditions

(Figure 5.2). We found that in the presence of an OPN neutralizing antibody, the colony formation ability was reduced under both normoxic as well as intermediate hypoxic environments. Thus, we can infer that osteopontin not only plays an important role in anchorage-independent growth in the normoxic environment but also has a significant role under hypoxic conditions of a tumor environment.

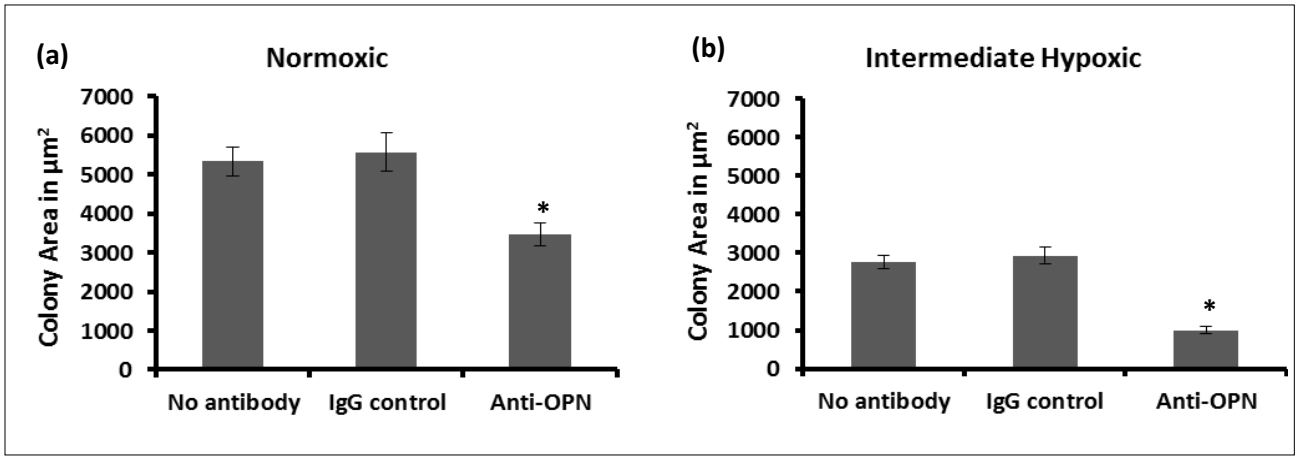



**Figure 5.2:** (a) Effect of anti-OPN antibody on MDA-MB-435 soft agar colony formation under normoxic environment. In the presence of OPN-neutralizing antibody, the colony formation ability reduces significantly under normoxic conditions. (b) Effect of anti-OPN antibody on MDA-MB-435 soft agar colony formation under normoxic environment. Under intermediate hypoxic conditions, cells treated with anti-OPN antibody, form colonies that are significantly smaller than untreated &/or control antibody treated cells. Thus, implying an important role of OPN for anchorage-independent growth under hypoxic tumor conditions. Colony area in $\mu m^2$ (mean ±S.E.) *Significance $p < 0.05$.

The colony formation ability of MDA-MB-435 cells showed a similar trend as MCF-7 cells as the conditions progressed from normoxic through intermediate hypoxic to hypoxic (figure 5.3).

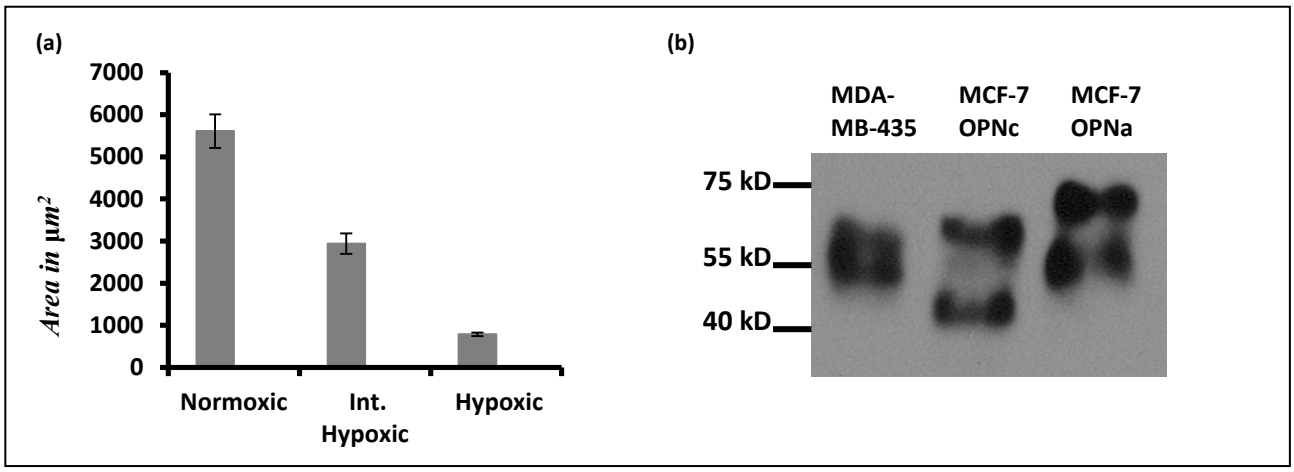



**Figure 5.3:** (a) Comparison of soft agar colony formation by MDA-MB-435 cells under early stage normoxic, intermediate hypoxic and late stage hypoxic environments. Colony area in $\mu m^2$ (mean ±S.E.) (b) Western Blot expression of endogenous OPN in MDA-MB-435 supernatants in comparison with MCF-7 OPNc and OPNa transfectants.

MDA-MB-435 cells produce all three splice variants of osteopontin - OPNa, OPNb and OPNc. To specifically analyze the role of OPNc, we performed soft agar colony formation assay using MDA-MB-435 cells in the presence of an OPNc-neutralizing antibody or a control Ig (figure 5.4). We found that the colony formation ability of MDA-MB-435 cells was significantly reduced after neutralization with an anti-OPNc antibody under both normoxic as well as intermediate hypoxic environments.

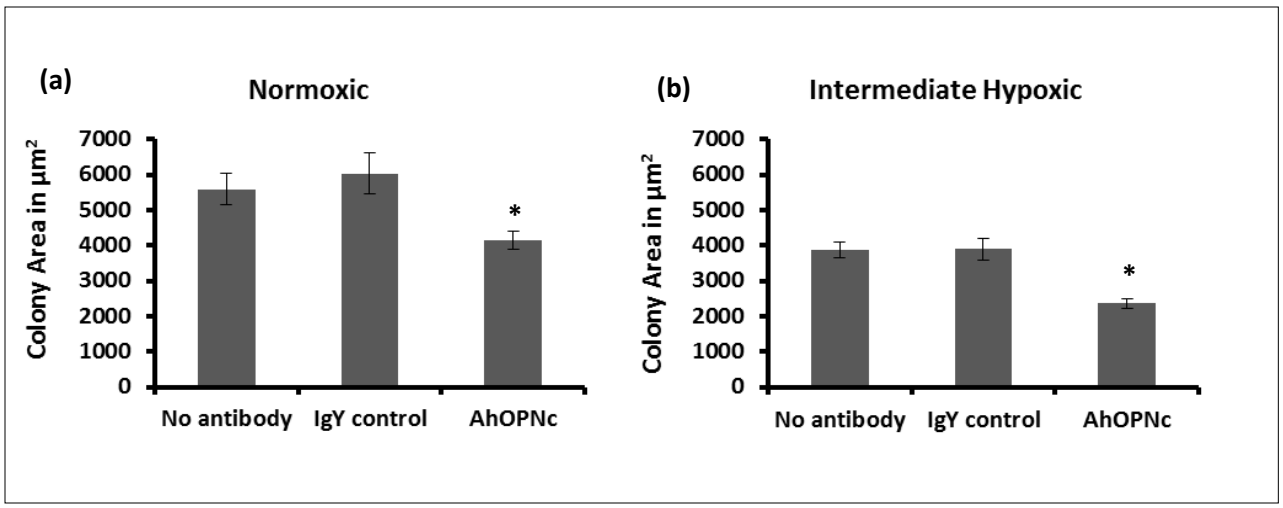



**Figure 5.4.** (a) Effect of anti-OPNc (AhOPNc) antibody ( AhOPNc, Gallus immunotech) on MDA-MB-435 soft agar colony formation ability under normoxic conditions. Cells treated with AhOPNc formed significantly smaller colonies than untreated or control IgY (IgY-010-NA, Gallus immunotech) treated cells. (b) Effect of AhOPNc antibody on MDA-MB-435 soft agar colony formation ability under intermediate hypoxic conditions. Under intermediate hypoxic conditions, the ability to form colonies reduced significantly for MDA-MB-435 cells, suggesting an important role of OPNc splice variant in hypoxic tumor environment. Colony area in $\mu m^2$ (mean ±S.E.) *Significance $p < 0.05$.

When MCF-7 OPN transfectants were treated with anti-hOPNc antibody, only the colony size for OPNc transfectants reduced significantly whereas the colony sizes for OPNa and empty vector transfectants were not significantly affected in the presence of the antibody (figure 5.5).

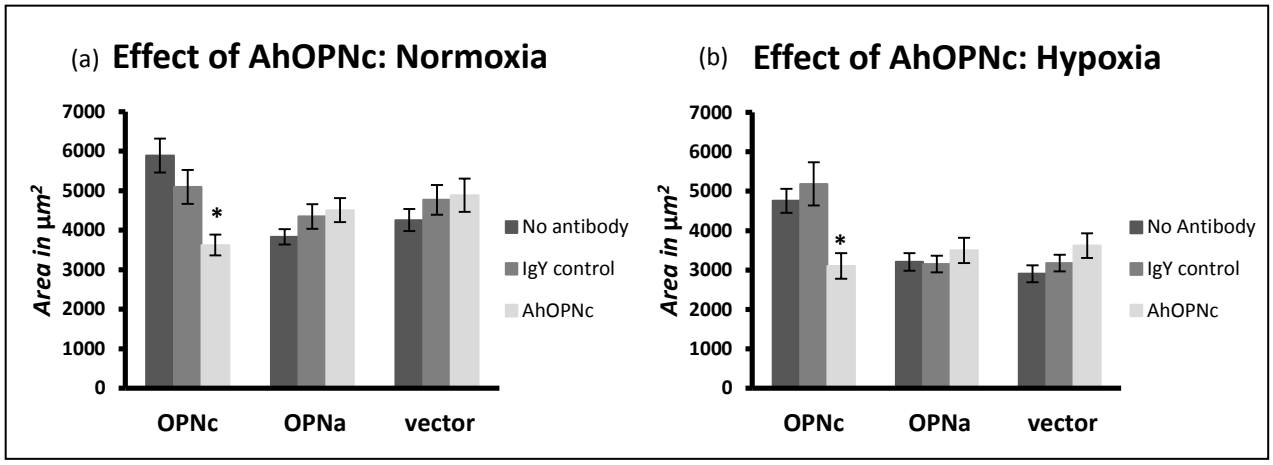



**Figure 5.5:** (a) Effect of anti-OPNc (AhOPNc) antibody on MCF-7 OPN transfectants' soft agar colony formation ability under normoxic conditions. Only MCF-7 OPNc cells treated with AhOPNc formed significantly smaller colonies than untreated or control IgY treated cells. (b) Effect of AhOPNc antibody on MCF-7 OPN transfectants' soft agar colony formation ability under intermediate hypoxic conditions. Under intermediate hypoxic conditions, the ability to form colonies reduced significantly for MCF-7 OPNc cells only, whereas colony sizes for OPNa and vector control remained unaffected. Colony area in $\mu m^2$ (mean ±S.E.) *Significance $p < 0.05$.

MCF-7 OPN transfectants also retained their ability of colony formation in methylcellulose (figure 5.6). OPNc transfected cells formed larger colonies than OPNa and empty vector transfectants under both normoxic and intermediate hypoxic environments.

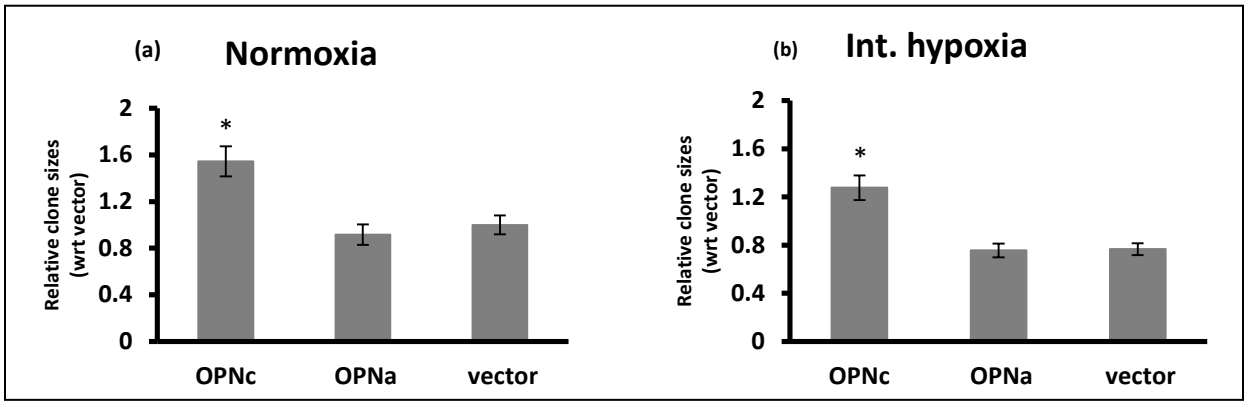



**Figure 5.6:** (a) Comparison of methylcellulose formation by MCF-7 transfectants: OPNc, OPNa and empty vector under early stage, normoxic environment. MCF-7 cells expressing OPNc formed largest clones. Colony area for each transfectant is normalized with respect to clone area of vector control under normoxic conditions (mean ±S.E.). Results are a representative of three separate experiments. (b) The ability to form larger clones was protected under intermediate hypoxic cancer conditions by cells expressing OPNc. Colony area for each transfectant is normalized with respect to clone area of vector control under normoxic conditions (mean ±S.E.). Results are a representative of three separate experiments. *Significance $p < 0.05$

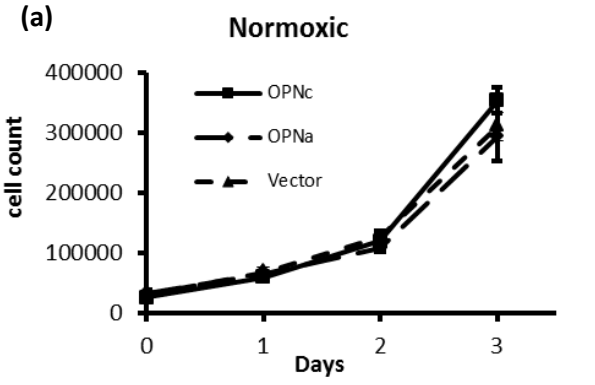
### 5.3.2. Cell Proliferation

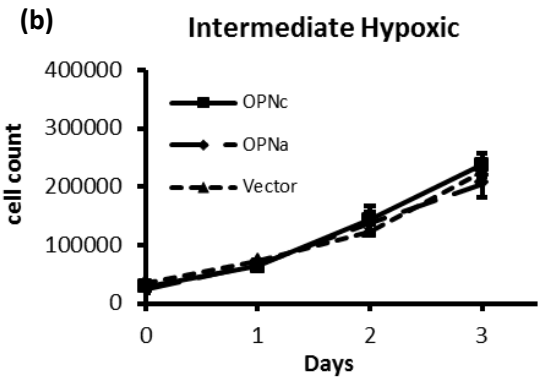
Anchorage-independent growth has two components: cell proliferation and anti-anoikis. Thus, our next step was to evaluate these properties in MCF-7 transfectants.

First, to analyze whether the ability of higher colony formation by OPNc transfectants is due to their higher proliferative ability over OPNa and vector, we performed a cell proliferation assay on plastic dishes under normoxic, intermediate hypoxic and advanced hypoxic conditions (figure 5.7). The cells were grown on plastic dishes under the three environments. Cell counting was done using an automated counter at four different time points: 0 h, 24 h, 48 h and 72 h.

Although the proliferation rate was less under the two hypoxic environments than under normoxic conditions, we did not obtain any statistically significant differences for cell numbers

on any day between MCF-7 transfectants: OPNc, OPNa or empty vector, under any of the three conditions.



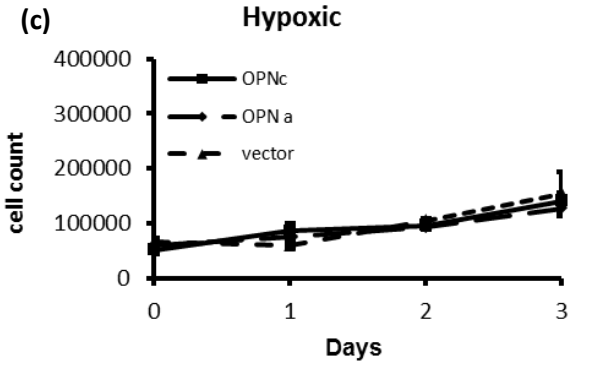




**Figure 5.7:** Cell proliferation assay for MCF-7 transfectants. The experiment was performed on plastic dishes in triplicates. There was no significant difference ($p < 0.05$) between cell numbers for OPNc, OPNa or vector on any day for the three conditions, (a) normoxic, (b) intermediate hypoxic and (c) hypoxic. However, the cell proliferation rate decreases as the environment progresses from normoxic to hypoxic.

### 5.3.3. Anchorage-Independent Survival

Next, to analyze whether expression of OPNc is important for anti-anoikis ability, we plated the three transfectants on a layer of poly-HEMA (avoids attachment of cells to solid surfaces, thus, the cells grow under deadhesion/anchorage-independent conditions) under both normoxic as well as hypoxic conditions. There were two controls: cells plated directly on the plastic surface of the 24-well plates served as a negative control for apoptosis; and cells plated directly on the plastic dishes with 700 μM hydrogen peroxide served as a positive control for apoptosis (data not

shown). The cells were stained with propidium iodide 96 h after plating and were analyzed for cell cycle stage by flow cytometry (figure 5.8).

In this case, although we did find that the hypoxic environment neither increased nor decreased the apoptosis as compared to the normoxic environment but OPNc transfectants are significantly protected from anoikis over OPNa and vector transfectants. The negative control for anoikis, that is, the transfectants plated directly on plastic dishes, did not show any significant differences for percentage of cells undergoing apoptosis.

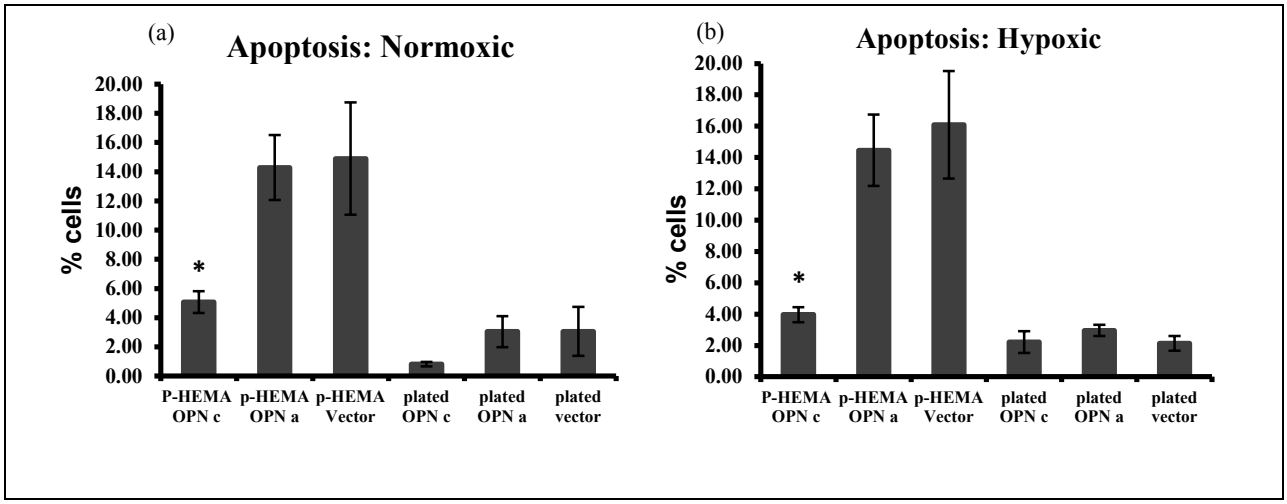



**Figure 5.8:** This figure shows the effect of deadhesion on cells undergoing apoptosis for MCF-7 transfectants. (a) Percentage of cells undergoing apoptosis (mean±S.E.) under normoxic conditions. (b) Percentage of cells undergoing apoptosis (mean±S.E.) under hypoxic conditions. The results shown are an average of 5 experiments under both normoxic and hypoxic conditions. There was no significant difference between individual transfectants, plated on poly-HEMA, under the two environments and also there was no difference between percent cells undergoing apoptosis for the three transfectants plated directly on solid plastic surface of the 24-well plates. *Significance $p < 0.05$

### 5.3.4. Effect of Oxidative Stress

Presence of OPNc seems to protect MCF-7 cells from undergoing peroxide-induced cell death at higher concentrations of $H_2O_2$ over OPNa and vector (figure 5.9).

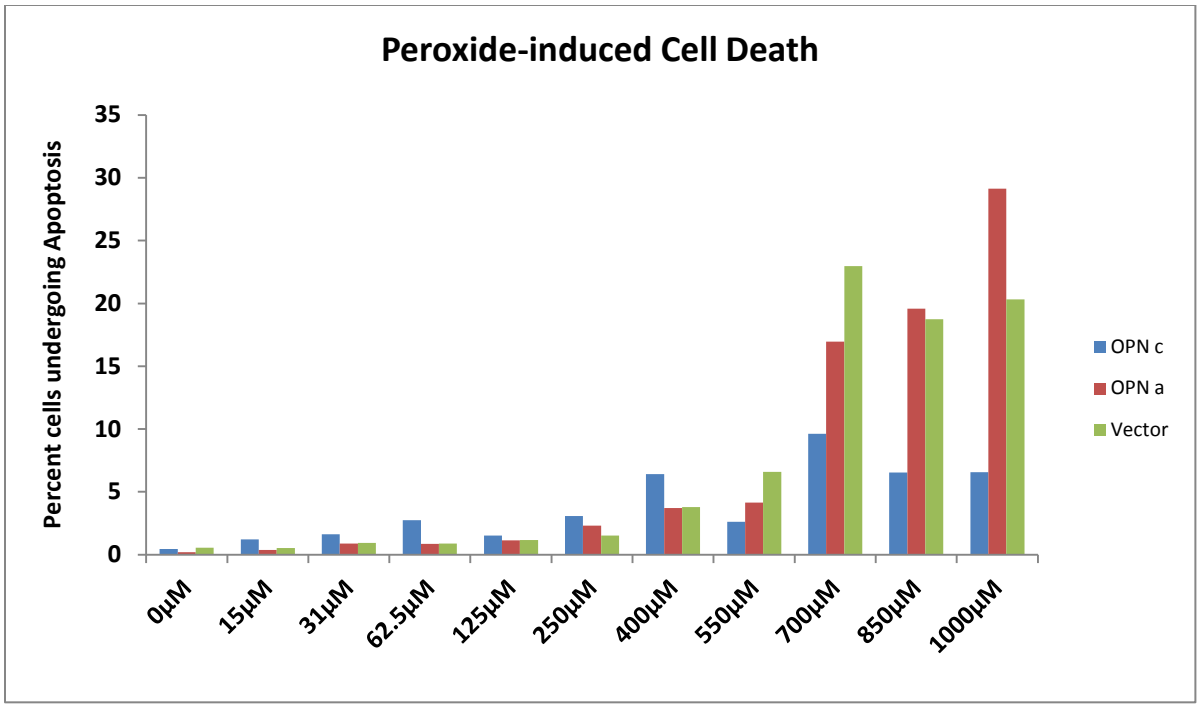



**Figure 5.9 Effect of H2O2-induced Apoptosis on MCF-7 transfectants.** This figure shows the effect of peroxide-induced cell death on MCF-7 OPN transfectants under normoxic conditions. The results were observed in one experiment after treating the cells with increasing concentrations of $H_2O_2$. There was no significant difference between individual transfectants at lower $H_2O_2$ concentrations, but with increasing $H_2O_2$ concentrations OPNc seemed to protect cells from peroxide-induced cell death.

### *5.4. Summary*

Out of all splice variants of OPN, OPNc has been known to have a higher anchorage-independent growth and survival activity for breast cancer cells under conventional cell culture conditions (He et al., 2006). Under hypoxic environment of advanced stage tumors, OPNc splice form maintains this phenotype of higher colony formation and better cell survival over OPNa and vector control even though the overall colony formation ability decreased under hypoxia for all cell types. Cell proliferation rates were similar for all the three transfectants under both conventional cell culture conditions as well as advanced hypoxic tumor microenvironments. Both OPN and OPNc neutralizing antibodies had an effect on soft agar colony formation. In the presence of anti-pan OPN antibody, the colony formation abilities for both OPNa and OPNc was reduced, while anti-OPNc antibody only affected the colony formation ability of MCF-7 OPNc

cells. OPNc expressing cells were significantly protected from anoikis under both normoxia and hypoxia. In conclusion, no hypoxia-specific phenotype was observed.

# Chapter 6
# Conclusions

# 6. Conclusions

### *6.1. Osteopontin Promoter Polymorphisms in Breast Cancer Aggressiveness*

Osteopontin promoter SNPs may encounter somatic mutations. The SNP at -443 site is important for the prediction of breast cancer aggressiveness. The T-allele at this site is associate with higher grade, ER- and PR- tumors. Our results corroborate that the osteopontin promoter SNPs -443 (rs11730582) and -1748 (rs2728127), which form a haplotype, are important for gene expression and breast cancer aggressiveness. Also, promoter polymorphisms were found to be significant over coding region polymorphisms for the identification of more aggressive tumors.

### *6.2. Alternatively Spliced Tissue Factor in Advanced stage Pancreatic Ductal Adenocarcinoma*

asTF is strongly expressed in late stage tumors, not only in PDAC lesions but also in lymph nodes and adjacent tissues. Thus, it becomes necessary to identify the significance of such strong asTF expression under advanced, hypoxic tumor conditions. We found that asTF over-expression significantly upregulates the hypoxia gene CAIX. Overexpression of CAIX not only affects cell growth via neutralizing the intracellular acidic pH of cancer cells, but also in turn decreases the extracellular pH of cells, thus, affecting their motility to certain degree under hypoxia (figure 6.1).

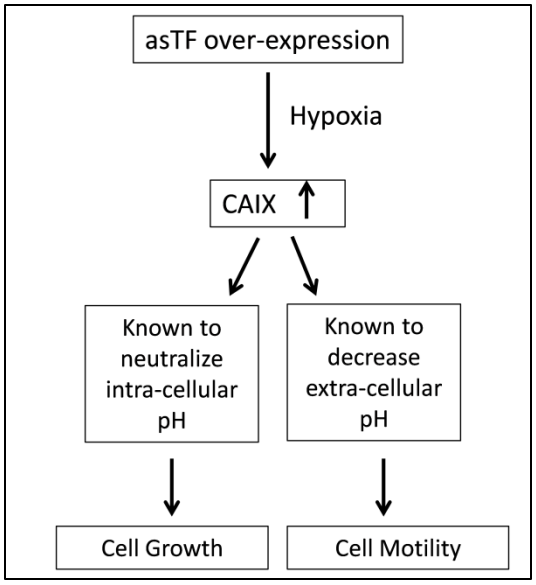



**Figure 6.1 CAIX Signaling.** Carbonic anhydrase IX is known to affect both intracellular and extracellular pH and may thus be significant in contributing to cancer cell growth and motility under hypoxic, acidic conditions of late-stage cancers.

# Chapter 7
# Future Recommendations

# 7. Future Recommendations

### *7.1. Aberrant Expression of Metastasis Genes and Tumor Progression*

Although the effect of polymorphisms in the promoter region of OPN gene has been studied comprehensively, much of the work still needs to be done in order to correlate these polymorphic sites with OPN expression levels and progression of various cancers. Single nucleotide polymorphisms may also fall within other regions of genes, including coding regions, non-coding regions (*cis*- and *trans*- regulatory elements, introns, etc.) and intergenic regions. We did identify and assess the significance of four coding region SNPs responsible for the non-synonymous mutations in the protein. More work needs to be done in order to establish the importance of synonymous mutations in this region along with SNPs at the sites that are vulnerable to protease cleavage, which may also affect alternative splicing in genes.

We have found a strong association of -443 site of SPP1 promoter with tumor grade, ER and PR status of the patients. Upon closer examination, T allele at this site was found to be more common in patients with a higher grade tumor, ER negative and PR negative status. Also, from literature we know that this site is a non-canonical binding site for *c-myb*, which is a downstream target of the signaling through estrogen receptor pathway. It may be implied that T-allele at this site may hinder the binding of *c-myb* at this site and may thus be responsible for a reduced response via estrogen receptor signaling. More ground work needs to be done in order to support this hypothesis, including how the alleles at -443 site regulate OPN expression and how they affect the binding of *c-myb*.

Furthermore, the expressions of various OPN splice forms with promoter polymorphisms in the SPP1 gene in breast cancer progression have not been well defined. We know that OPNc is

selectively expressed in cancer tissues and is associated with more aggressive breast cancers and a worse prognosis. It remains to be identified how the polymorphism, especially at-443 site, in SPP1 gene may regulate expression levels of OPNc and other OPN splice variants.

### *7.2. Alternative Splicing of Metastasis Genes and Tumor Progression*

The role of full-length tissue factor in the progression of various cancers has been well studied, but the alternatively spliced variant of tissue factor has not yet been fully evaluated for its significance in the progression of cancer. Since its discovery, asTF has been found to be very different when compared to its full-length form, in both physiologic and pathophysiologic functions. The signaling mechanisms and the integrin receptors that are engaged by asTF are very different from flTF.

Our study has described the importance of asTF in advanced pancreatic ductal adenocarcinoma where it is over-expressed. It remains to be elucidated the specific signaling by which asTF over-expression, and not basal asTF expression, causes significant CAIX upregulation under hypoxia. Figure 7.1 summarizes known interactions of asTF with various signaling pathways in transformed cells. The interplay of one or more of these signaling mechanisms might be responsible for the selective upregulation of CAIX under hypoxia. Also, the expression and effect of carbonic anhydrase XII, if any, needs to be elucidated.

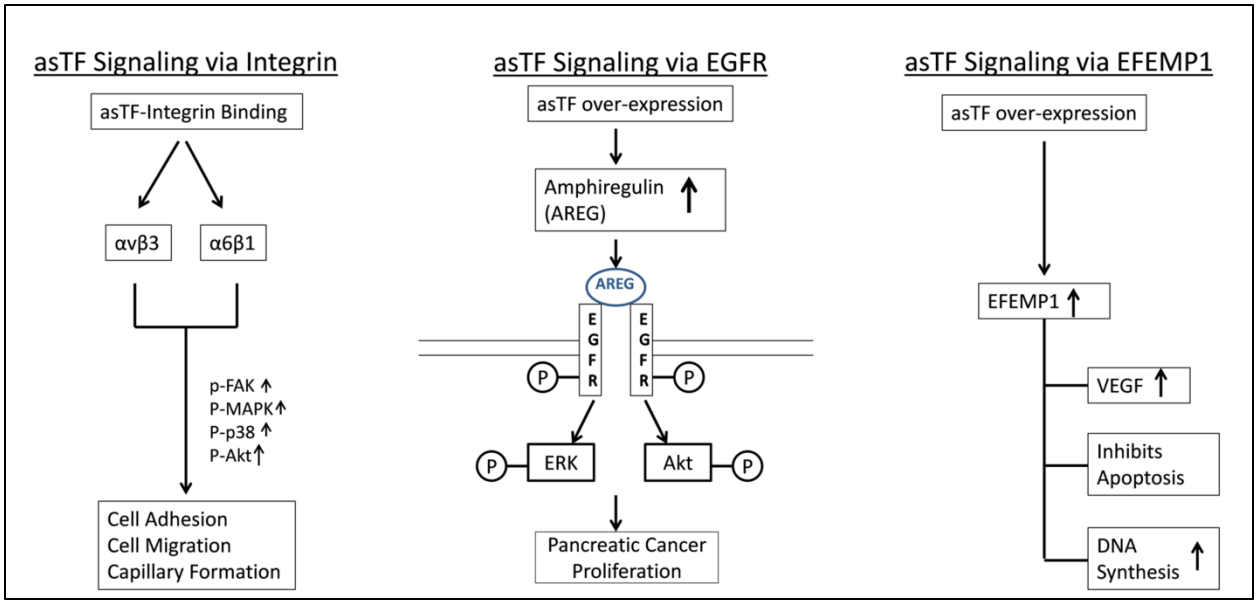



**Figure 7.1 Signaling pathways associated with asTF.** asTF, in contrast to full-length TF is known to exert its effect via a variety of different pathways. It is known to increase cell motility via its prominent interaction with integrin αvβ3 receptors (van den Berg et al., 2009), while disrupting the interaction of αvβ3 receptors with laminin. asTF is also known to increase cell proliferation in cancer via its indirect interaction with EGFR through amphiregulin (Yotsumoto et al., 2010) and is also known to affect other features of cancer progression, including angiogenesis and cell survival, via EFEMP1 pathway (Seeliger et al., 2009). One or more of these and other pathways may be involved in selective up-regulation of CAIX via asTF- over-expression even though both Pt45P1 and Pt45P1/asTF+ cells express similar levels of HIF-1α.

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